The population genetics of Festuca rubra on park grass

Thesis

The Population Genetics of *Festuca rubra* on Park Grass

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Abstract

An explanation for the ubiquity of sexual reproduction remains elusive although it is commonly suggested that sex and recombination facilitate a more rapid response to environmental change than clonal reproduction. If this suggestion is true then one would expect the frequency of sexual reproduction within populations to increase with rate of environmental change. The study described here investigates the potential correlation between mode of reproduction and rate of environmental change in red fescue, *Festuca rubra*, a clonal/sexual species of perennial grass. Populations are identified that have experienced contrasting histories of change over the past 138 years, and within which the ratios of clonally to sexually derived individuals have been determined. From these ratios the selection pressures on mode of reproduction in each of the *Festuca rubra* populations investigated are inferred. The unexpected discovery that *Festuca rubra* is represented by two sub-species at the main study site has also prompted investigations into the factors governing their relative frequencies and distributions. Since the sub-species represent different cytotypes within the red fescue aggregate, this has allowed for inferences to be made relating to their observed coexistence and the apparent overcoming of minority cytotype exclusion by the sub-species found at lower frequencies.

Chapter 1

Introduction

Dilution of the sexual female's genetic material by fifty percent each generation and the production of male offspring have been referred to as the 'two-fold cost of sex' (Maynard Smith, 1978). Yet the genetic recombination that occurs at meiosis is ubiquitous amongst plants and animals and suggests that there must be a significant advantage to sexual reproduction. However, the evolutionary function of recombination and the factors promoting it are far from understood despite decades of theoretical research (Hadany and Beker, 2003).

The costs and benefits of sexual reproduction: Fisher, (1930) postulated that recombination might accelerate adaptation to changing environmental conditions by facilitating the more frequent combination of beneficial mutations in individuals. Consequently, the ability of organisms to respond to temporal and spatial heterogeneity in environmental conditions has been thought to favour sexual reproduction (Mes *et al.*, 2002). What is more, in plants it might also allow individuals to escape from unfavourable environmental conditions (van Kleunen *et al.*, 2002) and provide means of long-distance dispersal into uncolonised habitats through the production of vagile seeds (Williams, 1975). However, over the long-term, assuming constant environmental conditions and no mutation, simulations show that the mean fitness of individuals comprising populations tends to decrease with recombination (Kimura, 1956; Lewontin, 1971). Similarly, whilst recombination provides a mechanism by which beneficial mutations may be brought together within a single individual, it also generates recombinants with an excess of deleterious mutations (Crow and Kimura, 1965). Bearing in mind the broad spectrum of genotypes that may be produced through the recombination of various genotypes within a population, and the resulting array of fitnesses (Otto and Barton, 2001), it is likely that only a certain sub-set of these recombinants will be suited to any specific

environmental niche. The remaining, less-fit genotypes may constitute a 'genetic load' on the population (Barton and Charlesworth, 1998; Briggs and Walters, 1997). Consequently, given that random mutations are more likely to be deleterious than beneficial (Kondrashov, 1993), recombination is likely to break up fitter combinations at a faster rate than new ones are created (Eshel and Feldman, 1970). Nonetheless, levels of genetic variance within populations are expected to be increased through sex and recombination, elevating the response of populations to changing selective pressures (Goddard *et al.*, 2005).

The costs and benefits of clonal reproduction: Clonal reproduction, on the other hand, can provide a means by which beneficial allelic combinations conferring local adaptation can be preserved (Mandujano *et al.*, 1998). Once optimal adaptation of genotypes to local environmental conditions have been achieved, there may be little advantage in the allocation of further resources to recombination each generation since whilst this would generate a new range of genotypes, only a sub-set of these would be likely to display high levels of fitness in the local environment. Of course, this is only true in benign environments where conditions are temporally stable. If the environment is changing rapidly, then the well-adapted genotypes of one generation may prove far less well adapted to environmental conditions encountered by the following generation. Given sufficient time, environments inevitably change. Hence, asexual reproduction is considered a short-term strategy facilitating rapid reproduction without the two-fold costs associated with sex (Peck *et al.*, 1998). Somewhat contradictorily though, certain species, such as bdelloid rotifers, are thought to have gone without sexual reproduction for millions of years (Butlin, 2002; Mark Welch and Meselson, 2000).

Amongst perennial plant species the majority can reproduce both sexually and clonally via vegetative means (Eckert *et al.*, 2003). This provides advantages not only in terms of enabling populations to inhabit both stable and fluctuating environments, within the constraints of interspecific competition, but also in facilitating the colonisation of new habitats and the maintenance of populations in a metapopulation context (Piquot *et al.*, 1998).

Since recombination offers a mechanism for the purging of deleterious mutations accumulated during clonal reproduction, mutation rate is likely to be an important factor affecting the relative rates of the two modes of reproduction in a sexual/clonal species. Similarly, the rate of adaptation amongst individuals within a population is likely to be directly related to the rate of environmental change. Consequently, theoretical models investigating the maintenance of sexual reproduction consider parameters that include the rate of environmental change and mutation rate (Butlin, 2002).

The influence of environmental change: The rates of sexual versus clonal recruitment within populations may depend upon ecological factors as well as relative production rates (Eckert *et al.*, 2003). In the clonal cactus *Opuntia rastrera*, clonal and sexual recruitment was shown to vary with habitat type and the associated effects of herbivory and shade cover on seedling survival (Mandujano *et al.*, 1998). If clonal and sexual propagules compete for sites in which to establish, then the rate of environmental change may also be an important factor in determining which type of propagule is more successful. If the environment is rapidly altering, a clonal propagule may represent a genotype that is no longer well-adapted due to significant environmental change since establishment of the propagules' parent. Hence, under

this scenario, sexually reproduced propagules may posses an advantage in terms of establishment.

However, where clonal propagules are able to out-compete seedlings, seed production may not necessarily be translated into recruitment and subsequent recombination (Silvertown *et al.*, 1993). Depending on the fitness of a genotype within an environment, one mode of reproduction may be more suitable than another. One can envisage that if a particular genotype generated through clonal reproduction exhibits reduced fitness as a result of environmental change, then persistent clonal propagation of this genotype may return a lower recruitment success than seen under prior environmental conditions. But if a strategy of sexual reproduction were adopted, then the genes of this individual may stand a greater chance of being recruited into the next generation, albeit dispersed amongst a number of novel genotypes.

Of course, the extent to which any particular genotype can remain optimised to local environmental conditions is dependent on the rate of environmental change. If environmental change is faster than the rate of adaptation, then genotypes may be limited to just 'keeping up' with the environment rather than achieving optimal fitness with respect to the conditions experienced at any one time. In this context the analogy of the Red Queen who has to run to stand still, from Lewis Carroll's 'Through the looking glass' has been drawn (Van Valen, 1973).

Fitness-associated recombination: Nonetheless, what might prove to be a successful strategy for one genotype may not be for another. A well-adapted clone might not need to go through recombination if its fitness in the local environment is still high. Consequently, Hadany and Beker, (2003) postulate that an optimal form of recombination, with respect to whole populations, is one where there is a higher probability for the breakdown of unfit genotypes relative to fit ones. In other words,

recombination would be optimal if it were negatively correlated with the fitness of the genotypes concerned. Hadany & Beker, termed this dynamic mode of recombination "fitness-associated recombination".

It is possible to imagine how, in colonising a novel environment, recombination might be favoured since it facilitates not only the physical movement of propagules into the new environment, but also rapid adaptation of genotypes to the environmental conditions therein. Once optimal genotypes have become established and levels of fitness increase, so clonal reproduction might be adopted, thus maintaining high levels of fitness in the greater majority of genotypes produced each generation.

Varying allocation to clonal and sexual reproduction: Intraspecific variation in reproductive allocation across populations has been shown to occur in several species. For example, Eckert *et al.*, (2003) identified variation in reproductive strategy across populations of the aquatic plant *Butomus umbellatus*. Similarly, studies on aphids and daphnia have revealed asexual lineages capable of producing males and thus generating potential interaction with sexual lineages (Innes and Herbert, 1988; Simon *et al.*, 1991). The proportions of these sexual and asexual lineages within such species have been shown to be influenced by environmental conditions as well as local adaptation (Halkett *et al.*, 2004; Simon *et al.*, 1999), although stringent selection may be necessary to induce an advantage to sex in asexual lineages as suggested for the unicellular alga *Chlamydomonas rienhardtii* (da Silva and Bell, 1996).

In plants, such variation in reproductive strategy can result from genotypic differences (cytotypic variation in the case of *B. umbellatus*), or from the plastic response of individuals to environmental factors (van Kleunen *et al.*, 2002). When adopted by populations experiencing differing rates of environmental change,

variation in reproductive strategy is likely to be reflected in the distribution of genotypes. Under more temporally stable environmental conditions, allowing for discernable levels of adaptation, clonal reproduction may be favoured and potentially translated into the dominance of one or a few genets (Janzen, 1977; Loveless and Hamrick, 1984). We might also expect an increase in clonality to be associated with reduced genetic diversity within populations. However, contrary to expectations this is often not the case and predominately clonal species regularly exhibit high levels of diversity (Alpert *et al.*, 1993; Eriksson, 1993; Kreher *et al.*, 2000; Yeh *et al.*, 1995). Nonetheless, where significant local adaptation has not been possible and both the production and selection of recombinant genotypes has occurred, the number of genetically identical individuals within a population is likely to be reduced.

Previous studies by Harada *et al.*, (Harada and Iwasa, 1996; 1997) set out to investigate the relative importance of clonal versus sexual reproduction in plant populations. Using spatial genetic data from their study populations they determined the probability of clonal identity via a novel statistical method. Whilst this method proved useful in determining the relative success of sexual versus clonal reproduction in circumstances where population demographic studies are difficult, it was nonetheless restricted in its assumption of equilibrium conditions, (Suzuki *et al.*, 1999). Specifically, these assumptions were that the species under investigation exhibit radially symmetrical growth of genets, that the study populations are in ecological equilibrium and that the habitats in which the populations exist are free from both spatial heterogeneity and variability in selection pressures.

Whilst these assumptions can be met in some plant species and habitats, e.g. the perennial woodland plant *Anemone nemorosa* (Holderegger *et al.*, 1998), they unfortunately do not fit others, including the populations that are the subject of this thesis.

The present study: In this study we have set out to investigate the consequences of variation in rate of environmental change on reproductive mode in the perennial grass species red fescue, *Festuca rubra*. Previous investigations into this sexual/clonal species within mountain grassland populations have revealed there to be no effect of initial colonisation conditions on the number of genets observed in later generations (Suzuki *et al.*, 1999). Instead, Suzuki *et al.*, considered short-term spatiotemporal variations to be most relevant in explaining the observed number of genets in contemporary populations.

Grassland habitats generally exhibit high levels of spatiotemporal variability (Silvertown *et al.*, 1988), making *Festuca rubra* an ideal organism for studying the ongoing effects of environmental change on mode of reproduction. Our chosen study site at Park Grass is an ancient meadow that has existed for over 250 years. The meadow has had differing fertilizer treatments applied to its' plots for nearly 150 years and exhibits heterogeneity in terms of rates of environmental change on different plots within the meadow.

Over the course of the investigations described in this thesis, an unexpected and previously unreported finding was made and subsequently confirmed by Mr Arthur Copping, a member of the Botanical Society of the British Isle who specialises in *Festuca* species and other members of the Poaceae. was that the study species, *Festuca rubra*, is in fact represented by an admixture of two sub-species on the Park Grass meadow, these being strong creeping red fescue, *Festuca rubra* ssp. *rubra,* and chewing's fescue, *Festuca rubra* ssp. *commutata*. The morphological analyses which lead to this finding had been prompted by the results of a preliminary investigation using dominant Inter Simple Sequence Repeat (ISSR) markers. Whilst the ISSR markers had proven unsuitable for further use on *Festuca rubra* due to a lack of reproducibility, they nonetheless identified a striking contrast in banding patterns

amongst a sample set of individuals representing populations from two of the Park Grass sub-plots; with each of the ISSR primers used, a noticeably greater number of bands were amplified in some of the individuals than in the others. It was speculated that these differing banding patterns were a consequence of the individuals concerned representing different sub-species with associated differences in chromosome numbers.

The study sub-species: Although morphologically extremely similar, ssp. *rubra* and ssp. *commutata* represent different cytotypes within the highly polyploid complex of red fescue species (6x–10x) and display contrasting tendencies in their clonal growth patterns; *F. r. rubra* is octaploid (8x = 56) and forms large, loosely aggregated clones through extensive rhizome growth (Grime *et al.*, 1988). *F. r. commutata*, on the other hand, is hexaploid (6x = 42) and, after several years of vegetative growth, forms dense clumps (Hubbard, 1984).

The wide range of habitats occupied by sub-species of the *Festuca rubra* complex is likely to be the result of ecological differentiation between the cytotypes (Grime *et al.*, 1988). Indeed, ssp. *rubra* may be able to exploit more spatially heterogeneous habitats than ssp. *commutata* since, in addition to pollen and seed dispersal, the extensive rhizome growth it exhibits provides a means of escaping from unfavourable habitats and colonising more amenable patches.

***Figure 1.1*:** Diagrammatic representation of the study species; *Festuca rubra* ssp. *rubra*.

Theoretically, we would expect the difference in ploidy to prohibit introgression between these two sub-species, since offspring resulting from a hexaploid/octaploid cross are likely to produce non-functional gametes as a result of unbalanced chromosome numbers. In discussing the difficulty of obtaining viable hybrid offspring from intercytotype matings, Marks (1966) coined the term 'triploid block' in reference to diploid and tetraploid individuals of *Solanum chacoense*. On the same basis it might be reasonable to view the sub-species of this study as reproductively independent units. However, the polyploid red fescue lineage has a phylogenetically recent origin (Charmet *et al.*, 1997) and at present may be more affected by recurrent

introgression than more distantly related species (Catalan *et al.*, 2004). Consequently, it will be important to consider the possibility of ongoing hybridisation and introgression when analysing genetic data relating to populations of these two sub-species on the Park Grass meadow.

The Park Grass Experiment: The meadow upon which these investigations are based is known as Park Grass and is part of the Rothamsted estate in Hertfordshire. Park Grass has existed as a meadow for several hundred years and the Rothamsted estate itself has been the site of agricultural research since the 1840s. The Park Grass meadow was incorporated into the estate's agricultural research programme in 1856 when Lawes and Gilbert began applying various manurial treatments to ½ and ¼ acre plots (see Figure 1.2) on the previously uniform grassland community to investigate the effects of fertilization on hay production. These manurial treatments proved to have differing effects on community assemblages and soil acidification. Combined with the application of two liming treatments every four years, started in 1903 and 1920 respectively, the plot soils had arrived at very different pH values by 1959 (Johnston, 1971).

In 1965 the plots were divided into sub-plots (*a*, *b*, *c* and *d*), and a new liming treatment applied such that sub-plots achieved and maintained pH values close to 7, 6, 5 and 4 respectively. Since pH of the control plot soils, representing the meadow as it was before treatments began, are around 5.0, an increase in soil pH to 7.0 on the *a* sub-plots was deemed too drastic to implement without first determining the consequences on the sub-plot *b* plant communities of raising pH to 6.0. Consequently, adjustment of soil pH on the *a* sub-plots was delayed for several years until the necessary assessments had been made.

Whilst many of the plots have now achieved the values of soil pH originally aimed for, to this date there are still some plots that are undergoing significant change in soil chemistry and acidity (Paul Poulton, Rothamsted Research Station; *pers. comm.*). However, to date, many communities have reached dynamic equilibrium (Silvertown, 1980).

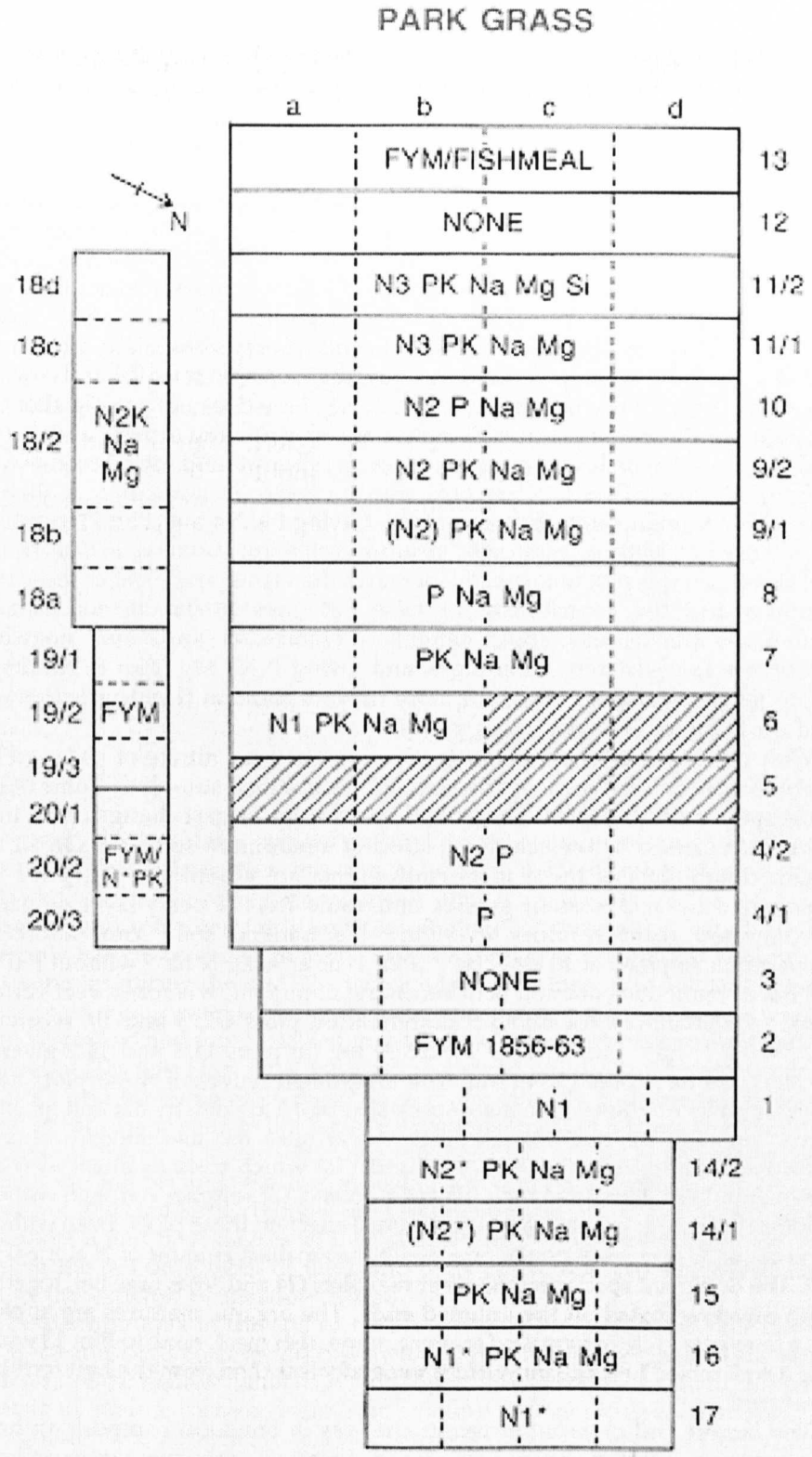


***Figure 1.2*:** Contemporary plot layout of the Park Grass Experiment (as provided by the Electronic Rothamsted Archive). Fertilizer and manurial treatments are as indicated for each of the sub-plots. FYM = farm yard manure. N1, N2 or N3 = ammonium sulphate applied at 48, 96, or 144 Kg per hectare respectively. N1* or N2* = sodium nitrate applied at 48 or 96 Kg per hectare respectively.

Assessment of community structure on each of the plots and sub-plots has been carried out meticulously every few years since the experiment began. The first hay cut is taken around the middle of June each year. To calculate the percentage composition of each species on the sub-plots a representative quantity of cut hay is taken from each and species abundances and biomass are recorded. As a result of these detailed records, we now have at our disposal an accurate and long-term data set with which to determine how community structure has changed across the plots and sub-plots of the Park Grass Experiment (PGE) over the past one and a half centuries

Primary objective: The aim of this study was originally based on the following hypothesis: the success of clonal versus seed recruitment in *Festuca rubra* is correlated with rate of environmental change.

However, since *Festuca rubra* is now known to be represented by two sub-species on the PGE exhibiting contrasting patterns of clonal growth and what are likely to be differing rates of spread, it has been necessary to refine and elaborate on our original hypothesis with two new hypotheses:

Hypothesis 1: The mode of recruitment in both *F. r.* ssp. *rubra* and ssp. *commutata* varies between sub-plots and is correlated with the amount of environmental variation experienced by a population. Greater environmental variation should result in more recruitment via sex and less from clonal reproduction.

Hypothesis 2: Since *F. r. commutata* has no rhizomes and reproduces sexually, its abundance should be positively correlated with the amount of environmental variation experienced by a population.

Further opportunities derived from the co-existence of two cytotypes: The unexpected discovery of the coexistence of ssp. *rubra* and ssp. *commutata* on Park Grass has provided an excellent opportunity for investigating the establishment and maintenance of mixed cytotype populations.

In assessing patterns of differentiation in cytotype frequencies amongst such populations we can elucidate the forces that govern the establishment of novel cytotypes. This in itself is fundamental to our understanding of plant speciation, since estimates of the number of angiosperm species which have undergone cycles of polyploidy at some point in their evolutionary history, whilst extremely variable, generally lie between 30 and 70%. Furthermore, Osborn *et al.*, (2003), explain that the more ancient polyploids (paleopolyploids) may have had their polyploidisation events obscured through genomic rearrangements. Given this, and the fact that even *Arabidopsis thaliana* (the angiosperm species with one of the smallest genomes) appears to have undergone polyploidisation leads the authors to suggest that perhaps all angiosperms have experienced polyploidisation events at some point in the past.

When new cytotypes arise within species, referred to as neopolyploids, they will undergo initial periods of establishment, often within or around populations of their already established progenitor cytotypes. Consequently, an understanding of how two cytotypes of the same species co-exist within the same population will provide a useful insight into the evolutionary forces governing the establishment of neopolyploids.

Evolution of polyploids and minority cytotype exclusion: The factors affecting the establishment, frequency and distribution of polyploids in mixed cytotype populations can be grouped into two categories. One involves environmentally dependent processes and considers the ecophysiological requirements of the cytotypes (Johnson

et al., 2003). The other involves environmentally independent processes governed by such factors as relative cytotype fitness, initial cytotype densities, rate of recurrent polyploid formation and inter-cytotype phenological variation.

Recent studies into the herbaceous perennial *Galax urceolata* found a mosaic distribution of cytotypes at the population level (Burton and Husband, 1999). The populations were shown to be strongly differentiated, tending either to be predominately diploid or predominately tetraploid. This, the authors explain, falls in line with theoretical predictions of cytotype frequencies within populations at equilibrium based on the premise that, under random mating, fitness in mixed cytotype populations is frequency-dependent (Felber, 1991; Levin, 1975). Under extreme circumstances fitness of the lower frequency cytotype may be so severely reduced, (as a result of insufficient pollen donors of the correct cytotype or saturation with inviable pollen from a majority cytotype (Levin, 1975)), that it is excluded from the population. This frequency-dependent process is known as minority cytotype exclusion and it is one of the principal hurdles a novel cytotype has to overcome in order to establish itself within a population.

Whether such frequency-dependent differentiation exists amongst *Festuca rubra* cytotypes on the Park Grass meadow may be dependent on their degree of ecological overlap; if there is sufficient deviation across the habitat mosaic in terms of those ecological factors affecting fitness of the cytotypes then we may find that habitat differentiation between them, should it occur, is instead the result of frequency *in*dependent factors. Any noticeable differences between the community assemblages of each population that might be associated with the observed cytotype distributions, or changes in their relative frequencies across populations, may help in identifying those evolutionary factors facilitating the establishment and persistence of the observed minority cytotype.

Secondary objective: Discovery of two *Festuca rubra* sub-species on the fine-scale habitat mosaic of the Park Grass meadow has provided an opportunity to investigate the establishment and maintenance of mixed cytotype populations and contribute to a better understanding of this fundamental, yet relatively under-investigated, mechanism of divergence. In line with the theoretical models of Levin (Levin, 1975) on 'minority cytotype exclusion' we have taken the opportunity to pose a third and final hypothesis:

Hypothesis 3: *Festuca rubra* will be represented by single cytotype populations on the separate sub-plots of the PGE.

Thesis outline: In Chapter 2, populations on the PGE that have experienced contrasting rates of environmental change are identified. This is achieved by analyses of species composition data along with detailed records pertaining to changes in soil chemistry for each of the 113 sub-plots of the PGE from 'The Electronic Rothamsted Archive' spanning the years of 1865 through to 2000. Several statistical methods are applied to determine rates of environmental change experienced by the plant communities of each sub-plot over this time period.

In Chapter 3, molecular techniques are employed with the aim of developing a method for determining the sub-species of each sample in the absence of morphological analyses and with access only to extracted nucleic acids. This involves the comparison of sequences from individuals known to represent both sub-species. The region focussed upon is the multicopy internal transcribed spacer region of the nuclear ribosomal DNA.

The relative frequencies of the two sub-species, or cytotypes, within each population are investigated in Chapter 4. Since the differing ploidies of the two cytotypes is known to be reflected in their nuclear DNA contents, flow cytometric methods have been employed to measure this trait in each of 180 samples collected from the study populations. Consideration of the observed frequencies and distribution of the cytotypes amongst the study populations enables the ecological factor(s) facilitating their coexistence and the overcoming of frequency-dependent processes by the minority cytotype to be determined.

In Chapter 5, nuclear and chloroplast microsatellite markers are employed to distinguish *Festuca rubra* genotypes collected from each of the study populations. Consequently, the relative contributions of clonal and sexual reproduction in each population will be estimated under the assumption that a genotype expressed by just a single individual within a sample-set represents the recruitment of a sexual propagule, whereas if two or more samples within a population express the same genotype, their presence is the result of clonal propagation.

Finally, the conclusions of this study are discussed in Chapter 6.

Chapter 2

Statistical analyses of community change over time

Introduction

As discussed in Chapter 1, contrasting rates of environmental change may impose differing selective regimes upon clonal/sexual species such that, at extremes, one mode of reproduction may be favoured over another. However, defining 'environmental change' even with respect to just one particular species is difficult due to the many factors, biotic and abiotic, that need to be taken into consideration.

Studies by Tuomisto *et al.*, (Tuomisto *et al.*, 2003), into spatial variation amongst pteridophyte and Melastomataceae communities across four regions of western Amazonia managed to account for a significant proportion of the observed variation in terms of geographic or environmental distances. Just ~25% of the observed community variation within the two groups remained unexplained, and might potentially be accounted for by unconsidered factors, such as drainage, micronutrient content of soils and environmental stochasticity. Nonetheless, even this successful study highlights the difficulties in accounting for differences in species compositions and abundances between communities.

Potentially, environmental change of relevance to *Festuca rubra* might be expressed in terms of change in the species' composition over time. Figure 2.1, below, shows how such change has occurred on six selected sub-plots of the Park Grass meadow since the experiment began. A casual assessment of the graph may lead one to conclude that the *F. rubra* population of sub-plot 10b has experienced considerable environmental change, whilst those populations of the five remaining sub-plots have witnessed relative stability. Indeed, the dramatic increase in composition on 10b between 1919 and 1948 is likely to be a consequence of the liming applications which increased the sub-plots' pH, enabling *F. rubra* to take advantage of the nitrogenous applications. However, the fact that such considerable changes in *F. rubra*

composition did not take place on the other sub-plots is not necessarily indicative of environmental or community stability. Equally considerable changes in composition may have occurred in other species with the likely consequence of imposing fluctuating selective pressures on the remaining species of each community, including *F. rubra*. The same case may be argued against using changes in total hay production as an indicator of environmental change. Whilst productivity on subplots may vary from year to year due to annual fluctuations in temperature, rainfall and other environmental factors (see Figure 2.2), when averaged over several years it may remain constant. Yet the relative contributions of individual species to such consistent overall productivity may change considerably over time.

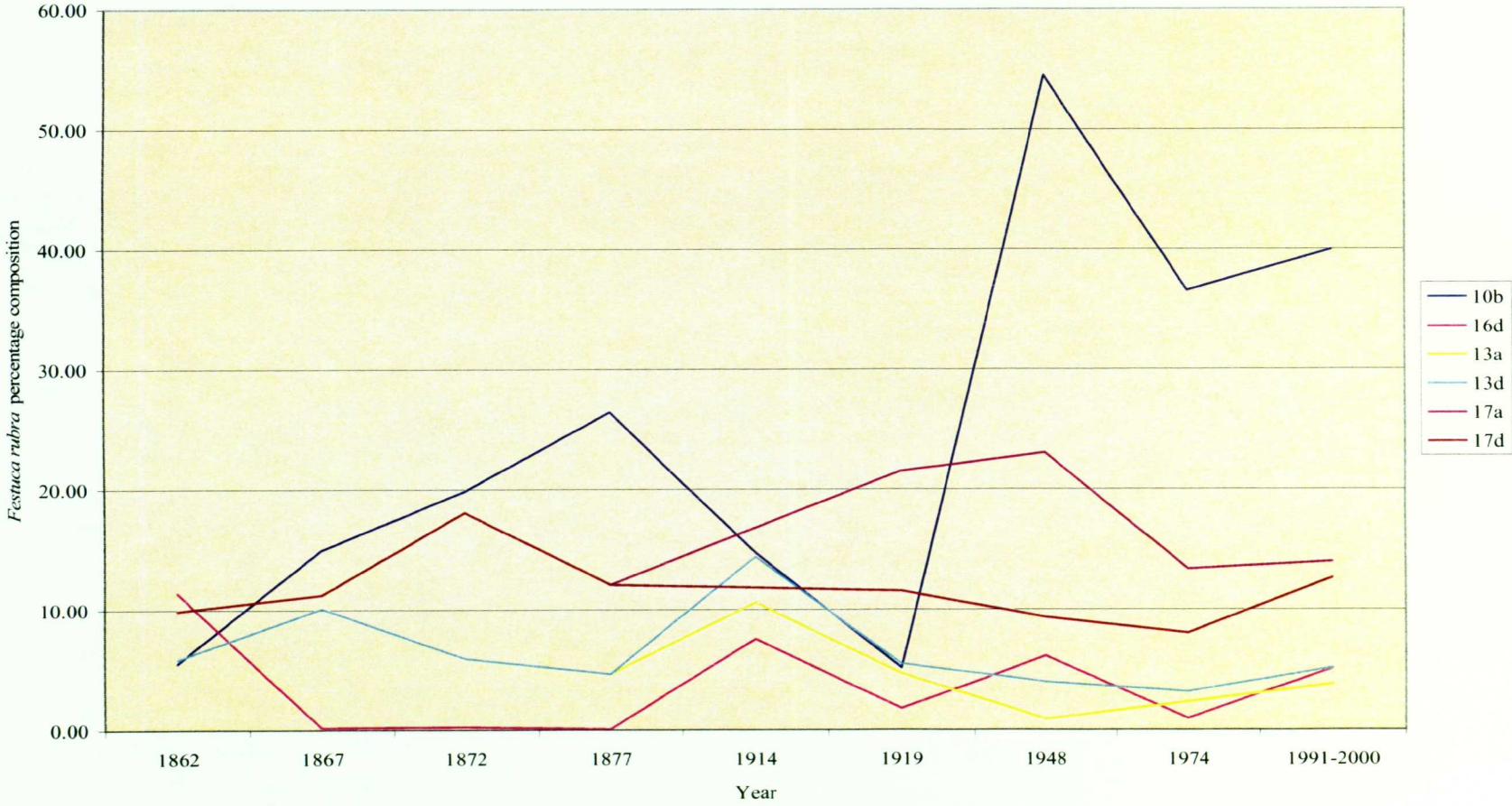


Figure 2.1: Change in *Festuca rubra* composition on selected sub-plots of the PGE between 1862 and 2000.

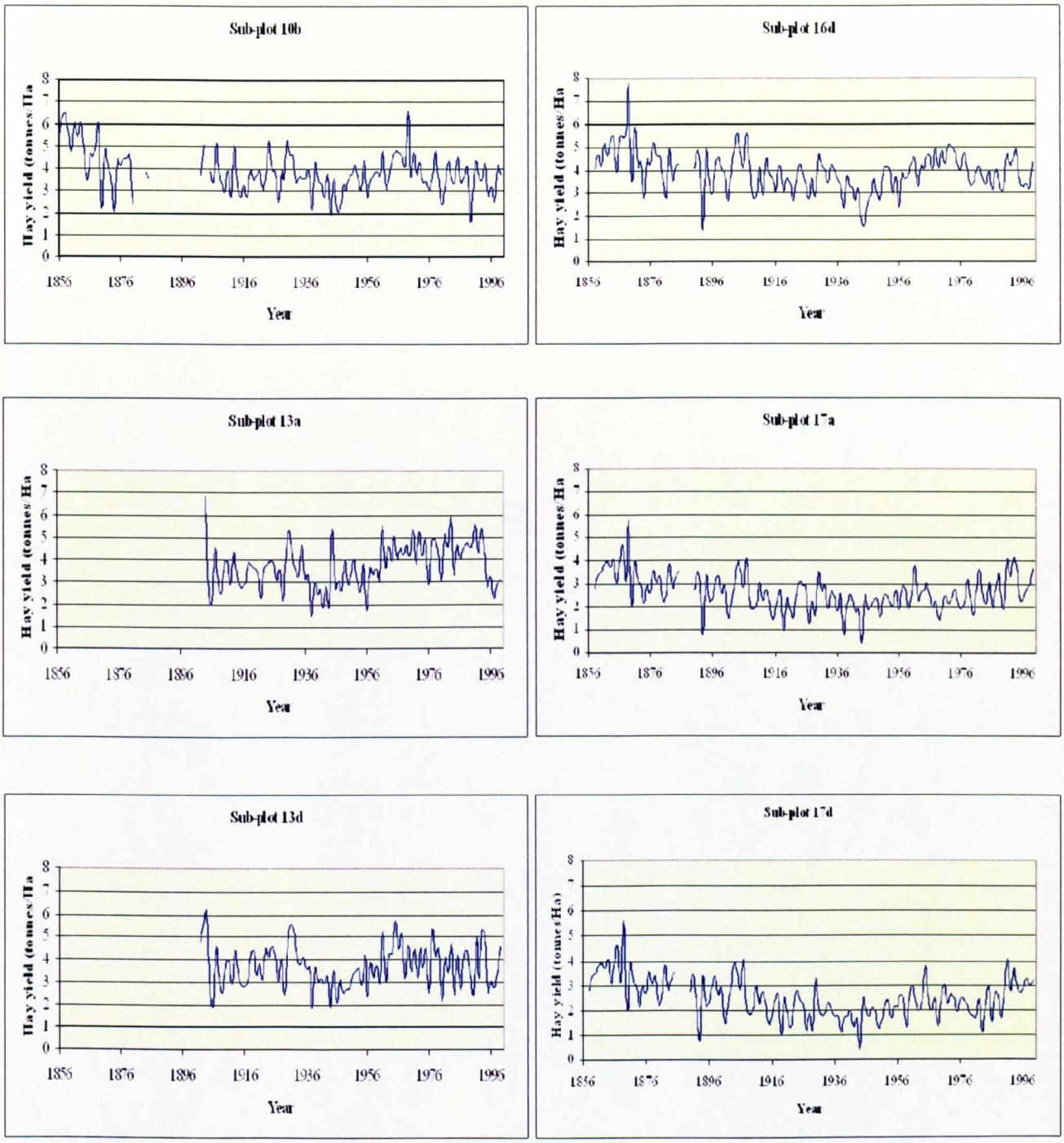


Figure 2.2: Change in productivity on selected sub-plots of the PGE between 1862 and 2000. Breaks in the data lines appear where information on hay productivity for particular sub-plots is unavailable.

Many environmental variables, such as fertilizer treatments, changes in soil chemistry and soil pH, have been recorded for each of the Park Grass sub-plots. Yet, numerous other factors are likely to affect *F. rubra* abundance, including levels of inter- and intraspecific competition, pollution tolerance of individuals and pathogen load. Such factors may indeed be of greater consequence to *F. rubra* abundance, in which case alterations in these will represent genuine environmental change relevant to *F. rubra* populations.

In the absence of data for each and every variable likely to affect *F. rubra* composition and abundance, an indirect method of quantifying change in all known and unknown factors is likely to be appropriate and exists in the form of measuring change in plant community structure over time.

Previous studies have shown that communities of the PGE have reached dynamic equilibrium. When considered over time frames of several decades, they exhibit stability in their characteristic proportions of grasses, legumes and other species (Silvertown, 1980), though percentage compositions of the individual species comprising each of these three groups may change relative to one another. However, whilst balance in the proportions of these three components is likely to be determined by different limiting factors relevant to each, compositions of the species comprising each component are likely to be governed by other factors. Hence, equilibrium in species compositions may be attained over different periods of time than equilibrium in the three major components (Silvertown, 1980). Consequently, whilst a community may have reached dynamic equilibrium, assessing fluctuations in species compositions is likely to represent a more sensitive measure of community change. Indeed, fluctuations in species compositions of plant communities have been related to changes in abiotic conditions (Cramer and Hytteborn, 1987; Prentice and Cramer,

1990; van de Maarel, 1981) since a community reflects the niches occupied by species therein, and the concept of niche considers the interactions of biotic and abiotic components (Prentice and Cramer, 1990).

The investigations described in this chapter set out to identify communities that have experienced contrasting rates of environmental change. This is carried out by considering temporal variation in species compositions within communities representing different sub-plots of the Park Grass Experiment.

Methods

Measuring change in species assemblages over time: The rates of environmental change experienced by populations of *F. rubra* on plots of the PGE are likely to be determined not only by the differing fertilizer applications, but also by the competitive interactions with other species found in each community as well as numerous other factors, both biotic and abiotic. Change in community structure, however, is likely to be a direct consequence of all components and hence should reflect ecologically meaningful differences (Tuomisto *et al.*, 2003). Consequently, assessing the similarity of communities representing individual plots at different time points over the last 140 years will provide a means of determining rates of change in community structure *and* environmental conditions.

However, three major factors concerning the analysis of species composition data require consideration before choices are made regarding the most appropriate statistical methods to adopt. Each of these has been given lengthy consideration and is discussed in detail below.

Quantitative or qualitative data? Species composition data have been determined for each plot on the Park Grass meadow since 1862. Whilst these data have not been collected every year, they have been collected at consistent time intervals. This involved cutting the crop, usually at the beginning of June each year, and making this into hay on the plot upon which it grew. Hay yields and botanical compositions were then recorded from sample sections of each plot.

Consequently, it is possible to access an extensive quantitative data set, held on the Electronic Rothamsted Archive (ERA), spanning over 140 years of community change. This quantitative data set provides a measure of each species' abundance on each plot as opposed to a simple binary index of presence or absence (qualitative data).

Quantitative data are more informative for purposes of community comparison; in a qualitative data set, a species which is found at high abundance on one plot, say at 95%, and low abundance on another, perhaps at only 5%, would be recorded simply as '1' or 'present' on both plots. Hence, information relating to the magnitude of differentiation in species abundances is overlooked.

In some circumstances, a qualitative data set is appropriate. For example, if one were to compare the fertilizer applications between plots of the PGE, '1' might represent the application of nitrates and '0' would reflect that nitrates have not been applied. Similarly, in situations where species diversity is so high, such as in rain-forest communities, one region may contain a completely different assemblage of species from another, hence qualitative data may again be adequate (Williams *et al.*, 1973).

However, it can be seen that the use of, or conversion to, a qualitative data set in assessing differences in species compositions when assemblages are relatively similar would lead to a loss of valuable information and resolution. Hence, when considering

appropriate methods for analysing the ERA composition data set, a decision was made to consider only those statistical metrics and analyses capable of handling quantitative data.

Exclusion or inclusion of species 'double-absences': A further consideration is the diversity of species within our data set. There are over ninety plant species recorded on the Park Grass meadow, many of which are rare. Furthermore, some species are absent from many plots and transient on others. Consequently, when we compare community structure of two plots, or that of a single plot at two points in time, there are likely to be occasions when one or more species are absent from both communities. In the field of ecological statistics, this is known as 'the double-zero problem'.

Species are thought to perceive optimum environmental conditions and to have unimodal distributions along environmental gradients (Huisman *et al.*, 1993; Whittaker, 1967). Because of the ecological preferences displayed by species, they are most likely to be found in habitats where these preferences can be met. Where conditions are optimal for a particular species, one would expect to find it at highest densities. Should conditions become progressively less favourable, one would expect the species to reduce in frequency, eventually becoming absent. In context of the large potential range of conditions that might be encountered within an environment, the optimum niche conditions for a given species are likely to be very narrow. Consequently, the presence of a species at two sites may be considered a strong indication of similarity in environmental conditions within these habitats. However, if a species is absent from two sites, it is at best tenuous to say that this is due to similarities between the sites.

A species may be absent from one site because conditions therein are above the optimal niche value and absent from a second site because conditions are below the optimum. Similarly, conditions may be below the optimum at both sites, or above the optimum at both sites. It is impossible to tell which of these scenarios is responsible for the double-absence of a species from a pair of sites and hence the inclusion of double-absences in community-comparisons may lead to artificially high values of similarity.

Statistical metrics and analyses which discount such double-absences, or double-zeros, are termed 'asymmetrical', whereas metrics which treat double-zeros in the same way as any other pair of values are termed 'symmetrical'.

The issue of whether the use of symmetrical or asymmetrical coefficients is appropriate when analysing data sets from diverse communities such as rain-forest and marine assemblages was much debated in the 1960's and 1970's (see (Field, 1969; Stephenson and Williams, 1971; Webb *et al.*, 1967). However, later studies tended toward the exclusion of double-zeros and the use of asymmetrical coefficients (Williams *et al.*, 1973).

The exclusion of double-zeros from comparisons of Park Grass floristic communities is deemed both appropriate and necessary for this investigation. Within the complex meadow communities of the PGE, numerous species are rare and absent from a large number of plots. Furthermore, a casual assessment of changes in species composition on plots over the past century reveals sporadic occupation of many plots by some species. As a result, the comparison of communities representing individual plots at different time points over the past century will contain a large number of double-zeros. The inclusion of these would suggest artificially high levels of similarity and would not reflect the true changes in community structure that have taken place.

Transformation of variables: A final consideration is the transformation of variables. In some ecosystems, communities are dominated by a relatively small number of abundant species. Other, less abundant species may also be present and often have the potential to provide informative diagnostic data pertaining to community structure. However, many statistical measures are sensitive to absolute values (Williams *et al.*, 1973) and consequently the diagnostic content of data on rare species may be lost due to the overwhelming influence of data from abundant species on the analyses employed.

Transformation is essentially the replacement of recorded values with another set of values. With respect to the species abundance values of the data sets considered here, transformation might be considered so that the values for each species are comparable with one another. However, transformation of the PGE composition data sets would arguably be inappropriate; should a method of transformation be applied, the complexity of the meadow communities coupled with the large number of rare and infrequent species therein may render the more subtle patterns of community structure susceptible to being obscured.

Though referring to rain-forest communities on a very different geographical scale from the meadow communities considered here, Williams *et al.*, (Williams *et al.*) suggest avoiding transformation or standardisation when measuring association among assemblages, giving similar reasoning as discussed above; the communities they examined contained large numbers of infrequent species whilst exhibiting high levels of within-site diversity. This, it was argued, made the relatively weak, large-scale patterns of the rainforest communities examined sensitive to standardisation. Moreover, the usual methods of transformation, applied to records of each species by the population variance or range, can simply reverse the emphasis such that measures

become more sensitive to the occasional presence of rare species (Jongman *et al.*, 1987; Williams *et al.*, 1973).

Consequently, with consideration given to complexity of the Park Grass communities, the number of rare and infrequent species encountered within them and the likelihood of large-scale patterns of community structure being overlooked, it was decided that the data in this thesis be analysed in its absolute form without transformation.

The application of selected methods of statistical analysis to Park Grass composition data: To summarise thus far, the most appropriate analyses and metrics for comparing floristic communities of the PGE are deemed to be quantitative, asymmetrical coefficients that do not standardise data sets.

For purposes of comparison three different metrics were employed. The 'City Block' and 'Mean Character Difference' metrics are both measures of similarity between sites (or communities). The third metric employed is known as the 'Total Proportional Dissimilarity' (TPD) metric. The two similarity metrics serve to express the ecological resemblance amongst two sites, whilst the dissimilarity metric is a complement to the similarity of two sites (Jongman *et al.*, 1987).

A further analytical method, known as 'Detrended Correspondence Analysis', (DCA), was also employed and served as an alternative method of comparing communities, the results of which could be cross-referenced with the three measures of calculated (dis)similarity.

Composition data were downloaded from the ERA for all those plots of the PGE whose populations contains sufficiently high compositions of *Festuca rubra* to allow sampling (more specifically, all those plots with *F. rubra* compositions $\geq 5\%$).

Composition data were also downloaded for two sub-plots known to have very low *F. rubra* abundance, but which were expected to exhibit the most widely contrasting rates of community and hence environmental change of any sub-plots pair on the meadow. These were sub-plots *10d* and *3d.*

Along with the three other sub-plots of plot *10*, *10d* has received inorganic fertilizers containing ammonium sulphate, sodium, potassium, magnesium and phosphorous since 1862. Thurston *et al.*, (Thurston *et al.*), explain that the greatest changes in sward structure are seen on those sub-plots of the PGE, that have received nitrogen in the form of ammonium sulphate as opposed to sodium nitrate, of which sub-plot *10d* is one. Coupled with this, sub-plot 10d has never been limed and has consequently undergone a dramatic decrease in soil pH to a current value of ~ 4.0. The species currently found on this sub-plot are few in number since a specialist tolerance of acidic soils is now a requisite.

Consequently, sub-plot 10d has been included in the species composition analyses; the measures of (dis)similarity for this sub-plot will serve a comparative function since the values will be known to represent truly elevated rates of community change against which those of other sub-plots can be compared.

Conversely, sub-plot 3d has acted as a control for the PGE as a whole, having had no fertilizers applied, organic or inorganic, or lime since the experiment began. The soil pH of this sub-plot has remained constant and we would expect it to have experienced the smallest amount of environmental change of all the sub-plots of the PGE. Since plant communities are dynamic and likely to change subtly even when environmental variables remain constant, the analysis of species composition data from sub-plot 3d will provide values for each of the dissimilarity metrics which can be taken to

represent those of a stable community experiencing low rates of environmental change.

Composition data are not available for the same years for each sub-plot of the PGE. Consequently, selected years were chosen spanning almost the entire duration of the field experiment, from 1862 to 2000. However, composition figures for each plot at the most recent time point are in fact averages of the values determined each year from 1991 to 2000. These average values of species composition were calculated to maintain the consistency in accuracy of the percentage compositions calculated over the course of the experiment; in 1991 the task of recording species compositions was passed over to the Imperial College research group and with this there was potential for differing techniques in assessing species compositions to have been employed. Consequently, it was decided that the use of values averaged over the 10 years of 'Imperial College data' would be advisable.

The resulting species composition data sets, representing selected plots at several time points each over the past 138 years, were analysed using DCA and the three measures of (dis)similarity referred to above, all of which are described below.

Detrended correspondence analysis: Developed by Hill & Gauch, (Hill and Gauch), DCA is possibly the most commonly used ordination technique (Palmer, 1993) and is regularly employed for the comparison of community assemblages since it can be used to infer environmental gradients from species composition data (Gauch, 1982; ter Braak, 1985). As with Principle Component Analysis, DCA arranges 'sites' (or in this case communities) along axes based upon the species composition data for each; it acts to reduce the large number of inter-correlated variables (species) to a smaller number of variables each of which explains a different portion of the observed

variation. The proximity of the sites, represented by points on a two dimensional graph, corresponds to their similarity in terms of species composition.

The de-trending function of DCA makes it preferable to PCA and other correspondence analyses as this serves to remove the false 'horseshoe' or 'arching' effect described by Hill & Gauch (1980), which is otherwise commonly encountered in the data. These mathematical artefacts are known to represent spurious second axes which are curvilinear functions of the first axis (Gauch, 1982) and possess no correspondence to any real structure in the data (Hill and Gauch, 1980). The detrending function ensures that the mean value of site-scores of the secondary axes, relative to any points along the first axes, is approximately zero (ter Braak, 1985). However, it should be mentioned that this de-trending function does not come without cost; DCA is a somewhat cruder method of ordination than correspondence analysis, from which it is derived, and whilst the de-trending function is beneficial for the reason given above, it also introduces inelegancies into computations (Palmer, 1993) and can lead to a loss of ecologically meaningful data (Pielou, 1984).

Nonetheless, the ordination diagrams (the two dimensional graphs depicting site similarity), provide a means of extrapolating inferences relating to the species composition data. Each site-point represents the central position amongst the species which occur within that site. Consequently, by comparing a site-ordination plot with the associated species-ordination plot it can be determined if a site and a species occupy similar positions. If this is the case then we would expect the species concerned to have a high abundance within that particular site. Conversely, if a site-point is at a very different position from that of a particular species-point on the ordination diagrams, we would expect that species to be rare or absent from the site.

In summary, "the expected abundance or probability of occurrence of a species decreases with distance from its position within the plot" (Jongman *et al.*, 1987).

As with each of the calculated dissimilarity metrics described below, DCA was applied to species composition data representing several separate years between 1862 and 2000 for each of a set of provisionally selected plots. Consequently, each of the resulting site-ordination diagrams displays site-points which represent the community within a single sub-plot, but at different points in time. Hence, the proximity of the site-points on the ordination diagrams corresponds to the degree of change in community structure over time, or indeed the rate of change.

Ordinations were carried out using the program CANOCO for windows 4.5 (ter Braak, 1985) with detrending carried out by segments. Rare species were down weighted, though transformation was not applied. Ordination plots were generated using CanoDraw for windows 4.0 (Smilauer, 1993).

City block: The name refers to the fact that, for two descriptors, the distance between two sites is the difference on the abscissa (descriptor y1) plus the distance on the ordinate (descriptor y2). The basic city block metric processes double-zeros in the same manner as any other value of descriptors and so, with consideration given to the double-zero problem discussed previously, the metric has been modified to exclude double-zeros from the data sets considered here.

Mean character difference: The "Durchschnittliche differenz", as it was first coined in German by the Polish anthropologist (Czenowski, 1909), was the second similarity metric employed. In line with the decisions made earlier regarding the specific

attributes of the statistical metrics employed in this study, this measure of community assemblage similarity has also been modified in our study to exclude double-zeros.

Total proportional dissimilarity: As with the other metrics employed in this investigation the TPD metric is a relative measure of difference between two communities or, for the purpose of this investigation, the species compositions of a single community at two different points in time. However, unlike the other metrics, TPD is a measure of the dissimilarity between two communities. Consequently, a high TPD value resulting from the comparison of two communities would imply similarity in their species compositions, whereas a low value would reflect dissimilarity. The metric is computed as the ratio of the sum of the minimum composition values for each species observed in the two communities, relative to the sum of the composition values of each species present in the communities. The TPD metric was designed and suggested by Bastow Wilson (*pers. comm.*).

Results and Discussion

Of those sub-plots examined using the DCA and dissimilarity metrics, six were identified as most contrasting in terms of relative community stability. These were sub-plots 10b, 16d, 17a, 17d, 13a and 13d and the following discussion refers to the differing rates of environmental change expressed in their floristic communities.

DCA results: The plots of communities and species in the ordinations represented in Figures 2.3a and 2.3b, below, are more evenly distributed in DCA space along axis 1 than axis 2. Nonetheless, the eigenvalues (quantities associated with variance accounted for by each axis (Sokal and Rohlf, 1995)) for the DCA show that axis 2 still accounts for 29.2% of the observed variation in species compositions between communities and 11.98% of the total inertia or variance in floristic data (see Table 2.1). When combined with axis one's eigenvalue of 40.9%, (16.66% of the total inertia) a total of 70.1% of the observed variation is accounted for. These high values are similar in strength to those obtained in a study by Vandvik and Birks, (Vandvik and Birks), which compared floristic data from vegetation types including grazed pasture and fenced infields in mountain summer farms in western Norway.

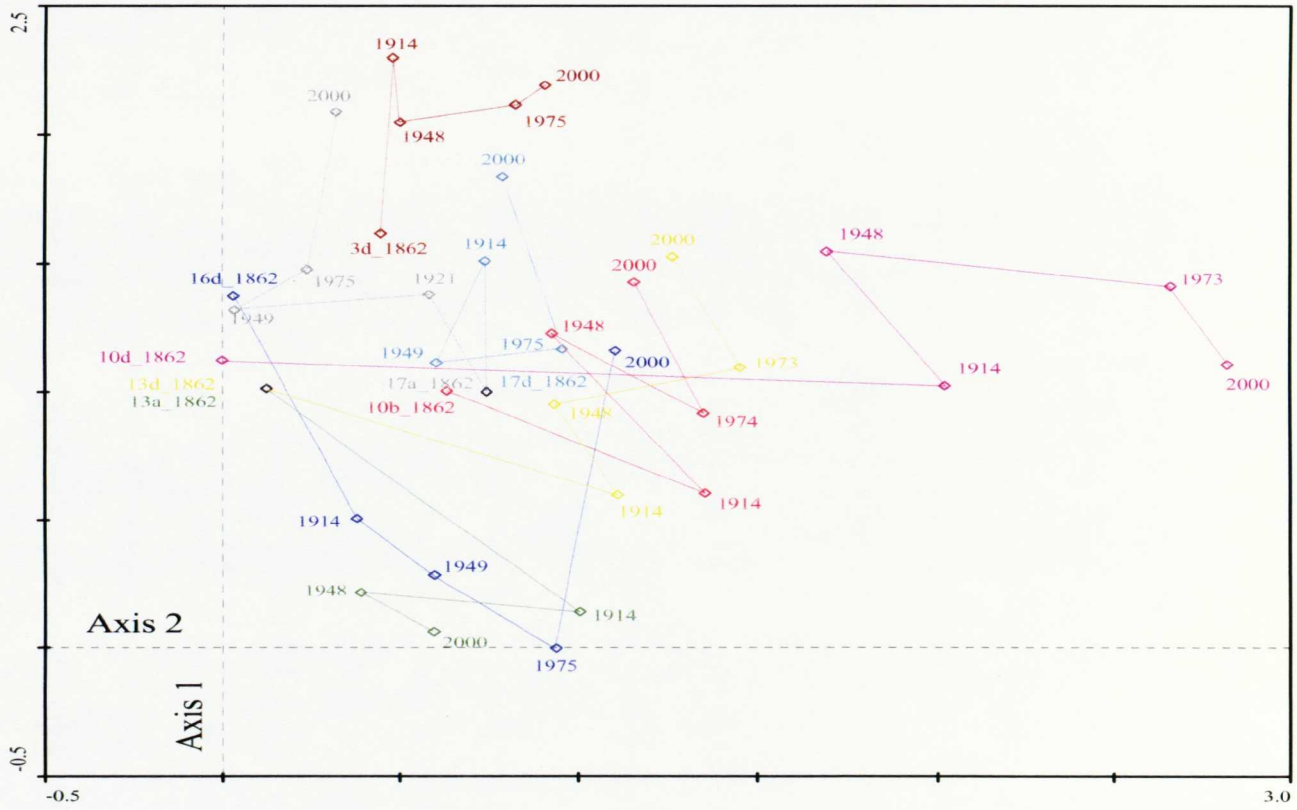
Like the Park Grass floristic data set, the mountain summer farm floristic data contained a large number of taxa, many zero values and a high compositional turnover. Consequently, Vandvik & Birks explain that, at 78%, the combined eigenvalues of axes 1 and 2 of their DCA is surprisingly high. Nonetheless, this value is only slightly greater than that associated with axes 1 and 2 of our DCA. What is more, the proportion of total inertia accounted for by axes 1 and 2 of our DCA is higher than that obtained from the mountain summer farm DCA.

In short, the variance explained by axes 1 and 2 in our DCA is high and, at over 70%, accounts for the majority of observed variation in species composition data between communities.

***Table 2.1*:** Eigenvalues and percentage total inertia associated with axes one to four of the detrended correspondence analysis.

Axes	**1**	**2**	**3**	**4**	**Total inertia**
Eigenvalues	0.409	0.292	0.154	0.094	2.455%
	16.66%	11.89%	6.27%	3.83%	

***Figure 2.3a*:** DCA Ordination of floristic communities representing eight sub-plots of the PGE at different time points between 1862 and 2000.



N.B: Axes 1 and 2 account for 70.1% of the observed variation in floristic data across communities. Also, plot and sub-plot communities are referred to using the contemporary names. Hence, for example, the community referred to as '*10b*' actually represents the community of plot '*10*' in 1862.

Figure 2.3a (above) shows the ordination diagram representing communities of eight sub-plots on the PGE at different time points over 138 years. Lines have been drawn between points representing communities of individual sub-plots in a chronological manner such that the amount of change in community structure between different times points on each sub-plot can be compared. This similarly allows for comparison of the amount of change in species compositions on different plots over the same time periods; the proximity of two points on the ordination diagram is proportional to the degree of similarity in the communities they represent. Consequently, the points representing the community of sub-plot *3d* at five different time points between 1862 and 2000, which are close to one another, suggest a limited amount of change in community structure over this period.

As already explained, sub-plot *3d* was included as a reference in the DCA. This sub-plot has acted as a control for the Park Grass Experiment as a whole, having received no fertilizer applications whatsoever since the experiment began. Consequently, the changes in floristic composition on this sub-plot have been small.

As expected, the community of control sub-plot *3d* has changed least out of all the sub-plots assessed via DCA. This is in stark contrast to sub-plot *10d*, the second and final control sub-plot used in the DCA. Sub-plot *10d* has received inorganic fertilizers since the beginning of the PGE and has experienced dramatic soil acidification as a result of withholding of liming treatments. Consequently, the community of sub-plot *10d* has undergone massive change, most notably over the 52 years spanning 1862 to 1914 and the 25 year period from 1948 to 1973. Only over the final time frame, from 1973 to 2000, does the rate of change appear to slow significantly to a modest rate comparable to that of the stable community of control sub-plot *3d*. Nonetheless, had

control sub-plot *10d* supported a contemporary population of *Festuca rubra*, it might have proved ideal for sampling, due to the elevated rates of change it has experienced. As intended, sub-plots *3d* and *10d* act as good reference points for our DCA. They exhibit widely contrasting rates of change in their respective community structures and for this reason, serve as useful comparative tools against which we can gauge the extent of change in communities on other sub-plots.

Sub-plot *16d* appears to have undergone considerable community change over the course of the PGE. It has experienced the same initial period of rapid change as seen on control sub-plot *10d*, though not to quite such a dramatic rate. However, in contrast to sub-plot *10d*, *16d* appears to have also gone through a period of extremely rapid change over the last time frame (1975 to 2000); the most dramatic change seen amongst all eight of the sub-plots considered.

Of the five remaining sub-plots considered, *10b*, *13d*, *17d* and *17a* appear to have experienced rates of change in community structure approximately half that observed on *16d* since the mid 1970's (and three to four times greater than that of control sub-plot *3d*). Unfortunately, sub-plot *13a* lacks a data point representing its' community in 1975 (the data are absent from the ERA). Nonetheless, the difference between the communities of sub-plot *13a* in 1948 and 2000 is minimal and comparable to that of the control sub-plot *3d* over the same period. Whilst it is possible that the unmeasured community of sub-plot *13a* in 1975 was more divergent from both the 1948 and 2000 communities, this would be in contrast to the observations on sub-plot *13d* which, other than liming, has received the same fertilizer applications.

The effects of environmental change over the past twenty five years are likely to be more easily detected, in terms of influence on mode of reproduction selected for in a contemporary population, than the effects of change several decades past. Previous

time frames are still likely to be relevant in considering rates of change, but it might be expected that their legacy, as reflected in the ratio of genotypes to individuals, will diminish as times goes by.

Consequently, it appears that sub-plot *16d* has experienced drastically elevated rates of change in floristic composition over the most recent time frame and indeed over the time span of the Park Grass experiment as a whole.

Sub-plots *10b*, *13d*, *17a* and *17d* have experienced more modest change over the two most recent time frames exhibiting rates that appear approximately intermediate to those seen on the control sub-plots *10d* and *3d*.

Finally, the floristic data representing the *13a* community has changed little over the single time frame available for this sub-plot, spanning 1948 through to 2000. This community appears to have behaved in a stable manner, similar to that of control sub-plot *3d*. As already mentioned, it is possible that, had species composition data been available for this sub-plot from the mid-seventies, it may have proven relatively divergent from that of the 1948 and 2000 communities, but this would be in contrast with the relatively minor changes that have taken place on sub-plot *13d* over this time period. Also, of these two sub-plots, it is *13d* that we would expect to have changed more rapidly due to the dramatic decrease in soil pH. Consequently, the small degree of change between the communities sampled from sub-plot *13a* in 1948 and 2000 is unlikely to mask more dramatic changes that may have occurred in the intervening years when compositions were not measured.

It is noteworthy that, other than sub-plots *13a* and *10d*, the changes in community structure on all other sub-plots appear to follow a common pattern over the last time frame (1973/4/5 to 2000); the communities each show an increase in their values/positions on first DCA axis of the ordination diagram in Figure 2.3a. Whilst

the DCA does not specify which of the environmental variables, or combinations thereof, each axis represents and indeed data relating to these was not incorporated into the analyses, it is still possible to speculate by cross-referencing with the species ordination diagram; species occupying the same area in DCA space as a particular community provide us with means of determining the environmental variables having greatest influence on structure of the community considered.

Ellenberg values: These values provide measures of the ecological tolerances of species, as estimated by Ellenberg, (Ellenberg), and any correlations in these values for species occupying similar positions in DCA space provide us with indications of the ecotypes represented by the communities under consideration. Values of five ecological variables are available. These are the **L**ight intensity required by different species, **M**oisture requirements, **R**eaction or acidity/lime tolerances, **S**alt tolerances and finally **N**itrogen or, more generally speaking, nutrient requirements.

Table 2.2 lists Ellenberg scores, for the five ecological variables, of eight species represented in the species-ordination plot (Figure 2.3b). The species listed are found in the uppermost area of the ordination plot where first axis values are high. If we compare these Ellenberg values with those of species found in the lower section of the species ordination plot, where the first axis values are low (see Table 2.3), we can see that whilst the average values for variables **L**, **M**, **R** and **S** are very close across the two groups of species, the **N** values are significantly different.

The mean Ellenberg **N** value for the eight species in Table 2.2, which are found at the higher end of the DCA first axis, is 3.8. This is indicative of species found on nitrogen or nutrient deficient soils (Ellenberg, 1988). However, the mean **N** value of the species listed in Table 2.3, which occupy the lower end of the DCA first axis, is 6.9.

This value is common amongst species most abundant on nutrient rich soils (Ellenberg, 1988). Consequently, the change in communities on sub-plots 10b, *16d*, *17a*, *17d*, *13d* and *3d* over the past quarter of a century appears to be toward the exclusion of nutrient-loving species and inclusion of herbaceous species tolerant of nutrient poor soils.

In light of the fertilizer treatments applied to the Park Grass meadow it seems unlikely that any of the sub-plots, other than those left unfertilised, are genuinely nutrient poor, although, to a degree, the annual removal of hay may confound such expectations. Nonetheless, it might be suggested that the exclusion of nutrient-loving species on sub-plots is a result of the depletion of trace nutrients, not being replenished through fertilization, by the increased hay yields associated with successive fertilizer applications. Lack of availability of such trace nutrients may now be restricting growth of nutrient-loving species and bringing about associated changes in community structure.

Figure 2.3b: Species-ordination plot generated by the application of DCA to species composition data representing eight sub-plots of the PGE at different time points between 1862 and 2000.

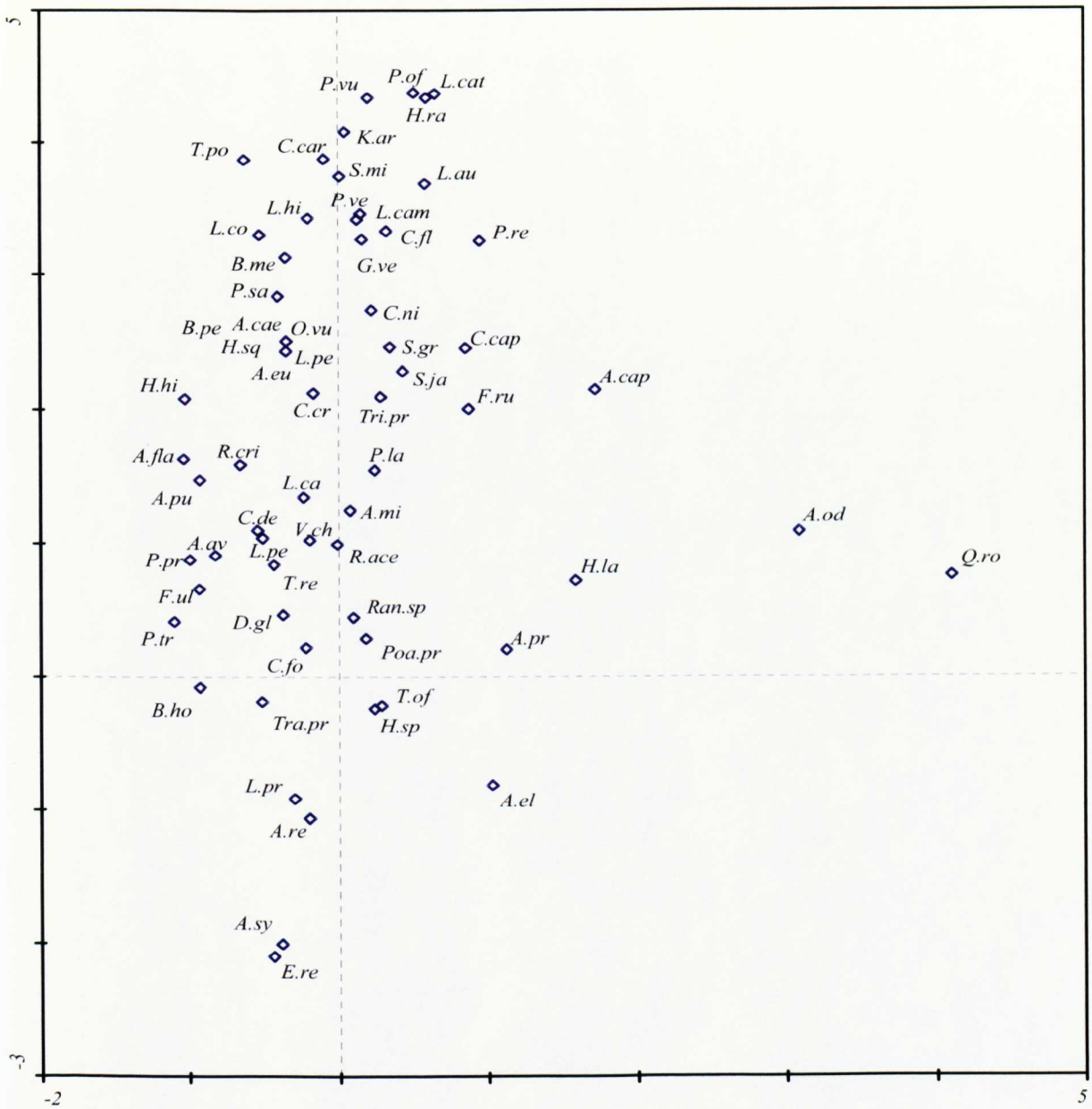


Table 2.2: Ellenberg scores for eight species found at high values along the X axis of the DCA species ordination plot.

Species	Light **L**	Moisture **M**	Reaction **R**	Nitrogen **N**	Salt **S**
Pulmonaria officinalis	4	6	8	7	0
Linum catharticum	7	-	7	2	1
Primula vulgaris	6	5	7	5	0
Hypochaeris radicata	8	5	4	3	1
Knautia arvensis	7	4	-	4	0
Carex caryophyllea	8	4	-	2	0
Sanguisorba minor	7	3	8	2	0
Leontodon autumnalis	7	5	5	5	0
Mean	**6.8**	**4.6**	**6.5**	**3.8**	**0.3**

- = Ellenberg scores not available.

Table 2.3: Ellenberg scores for seven species found at lower values along the X axis of the DCA species ordination plot.

Species	Light **L**	Moisture **M**	Reaction **R**	Nitrogen **N**	Salt **S**
Elytrigia repens	7	-	-	7	0
Arrhenatherum elatius	8	-	7	7	0
Anthriscus sylvestris	7	5	-	8	0
Ajuga reptans	6	6	6	6	0
Lathyrus pratensis	7	6	7	6	0
Tragopogon pratensis	7	4	7	6	0
Heracleum sphondylium	7	5	-	8	0
Mean	**7**	**5.2**	**6.8**	**6.9**	**0**

- = Ellenberg scores not available.

(Dis)similarity metric results: The results of the statistical metrics are graphically presented in two different formats; a line graph format plotting change in each metric's value for each sub-plot over time and a block graph format displaying the average metric value for each sub-plot over the 138-year period over which composition data were analysed.

Each of the metrics were applied to composition data from several time points between 1862 and 2000 representing the eight sub-plots previously considered, including the control sub-plots *3d* and *10d*.

The values resulting from application of the (dis)similarity metrics reflect the level of similarity, or dissimilarity, between each pair of chronologically consecutive communities where each 'series' of communities represents an individual sub-plot between 1862 and 2000.

Three metrics were employed for analysing the species composition data both for comparative purposes and because it was expected that different metrics would display varying levels of sensitivity to the species composition data.

By looking at the block graphs in Figures 2.4, 2.5 and 2.6, below, which represent the average change in community composition across all time frames on each sub-plot as determined by each metric, it is apparent that the results of the Mean Character Difference (MCD) metric are most closely in line with our expectations.

As in the DCA, the control sub-plots *3d* and *10d* serve as lines of reference against which the results from other sub-plots may be compared, and as expected control sub-plot *3d* is shown by MCD metric analysis to have experienced minimal change in community structure whilst control sub-plot *10d* is shown to have undergone dramatic change.

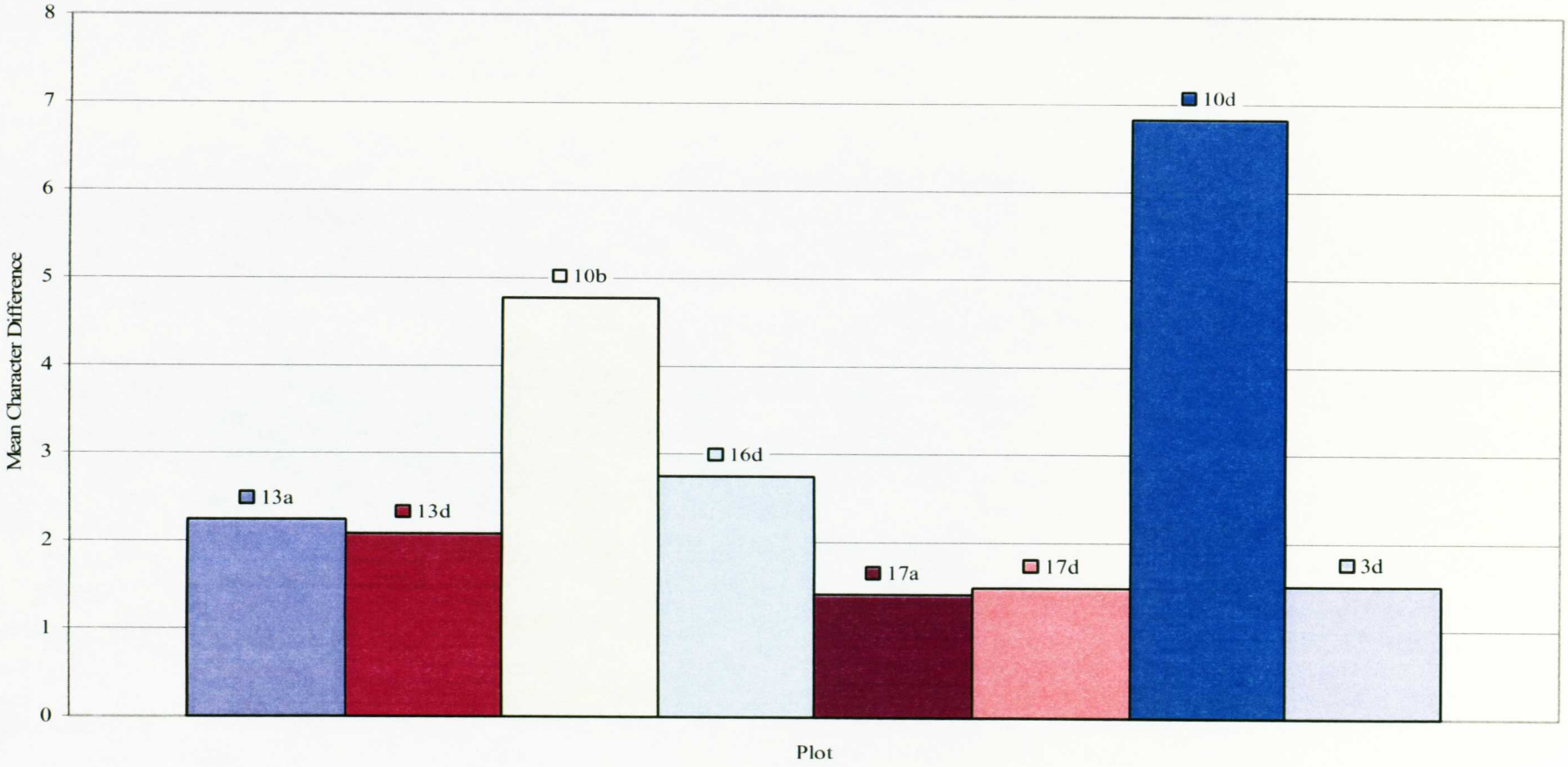


Figure 2.4: Average amount of community change between chronologically consecutive communities on individual sub-plots between 1862 and 2000, as estimated using the Mean Character Difference metric.

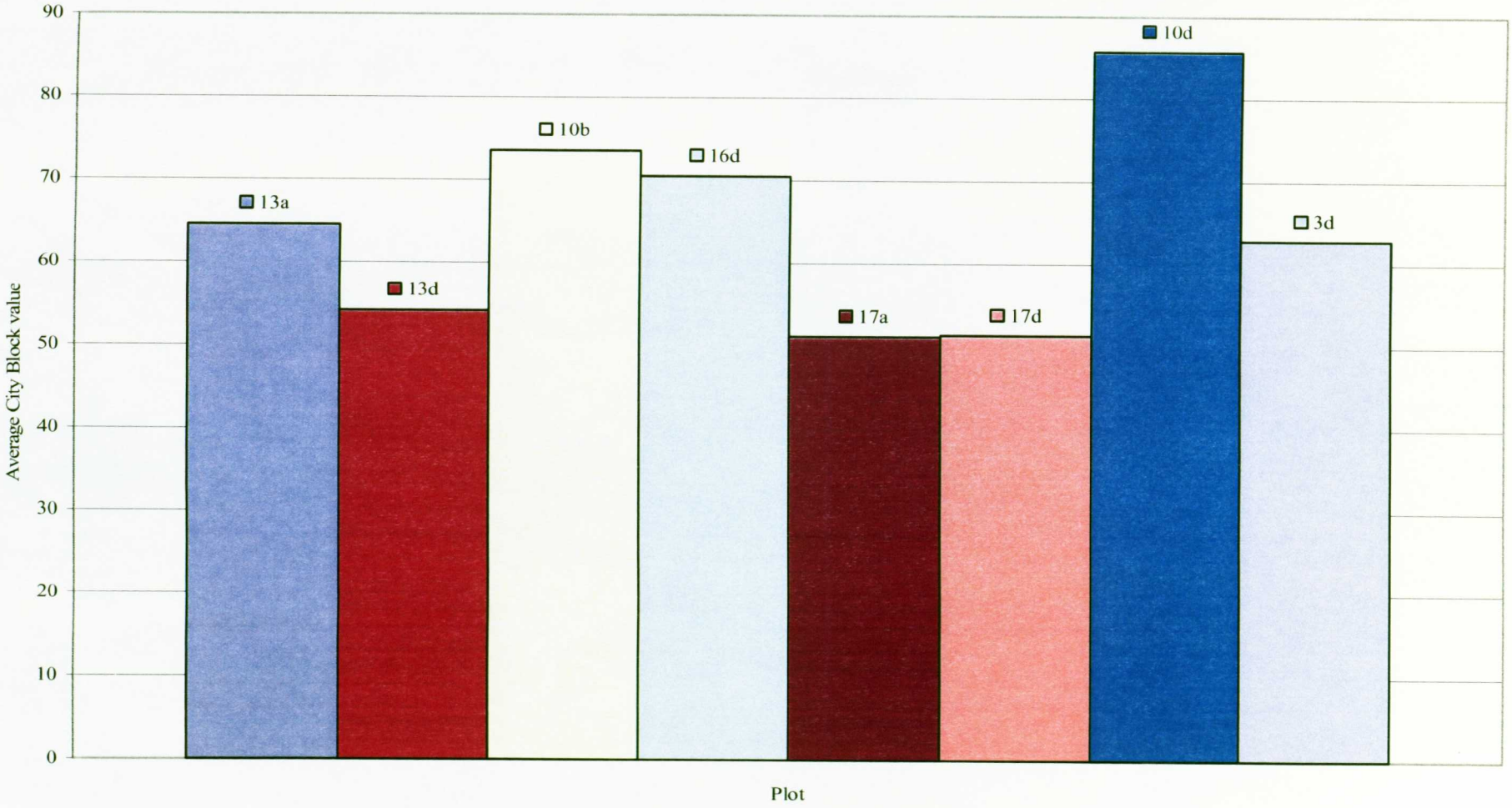


Figure 2.5: Average amount of community change between chronologically consecutive communities on individual sub-plots between 1862 and 2000, as estimated using the City Block metric.

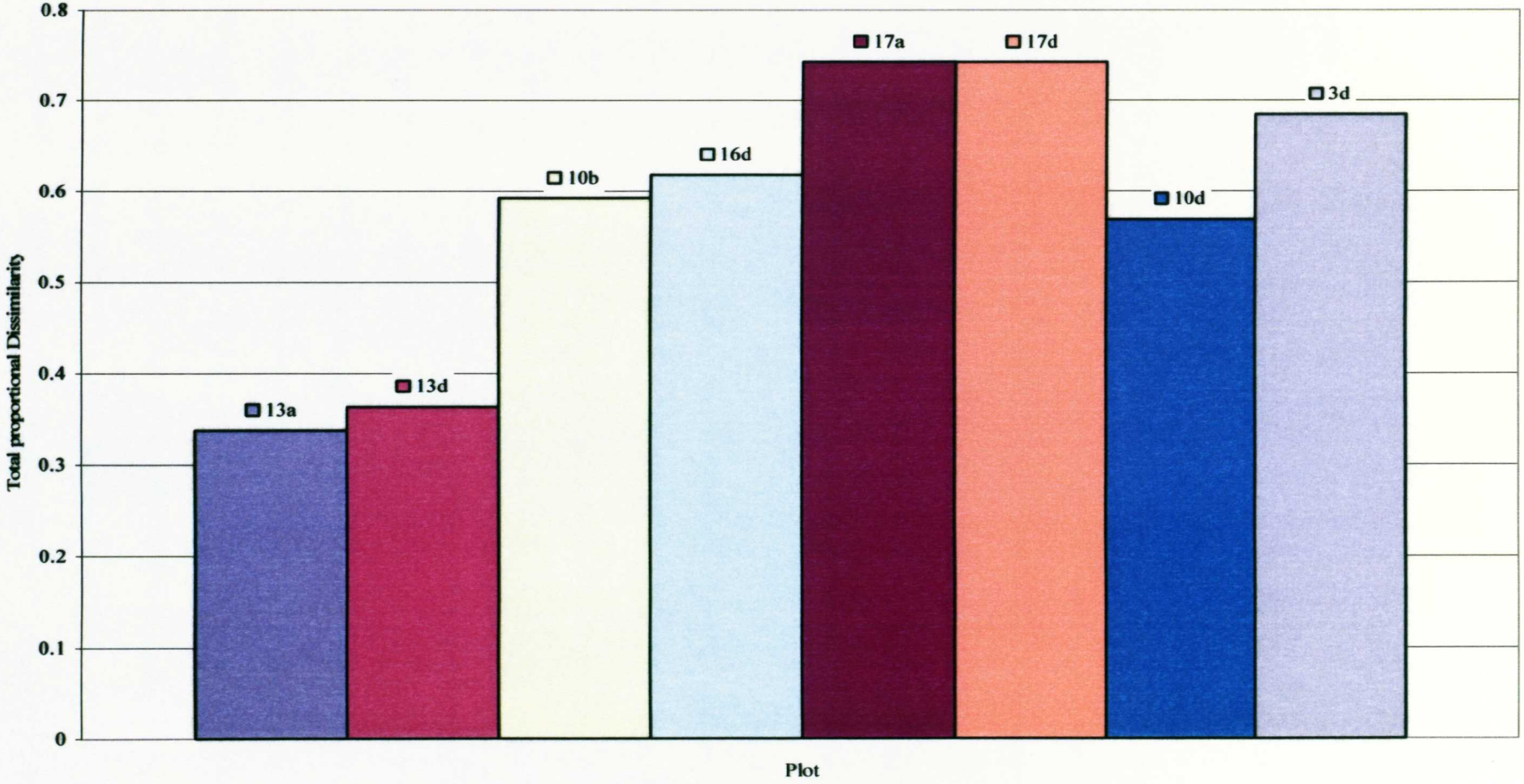


Figure 2.6: Average dissimilarity between chronologically consecutive communities on individual sub-plots between 1862 and 2000, as estimated using the Total proportional Dissimilarities metric.

Nonetheless, the status of these sub-plots as strong outliers in terms of relative rates of community and environmental change is not expressed in the results from the City Block or Total Proportional Dissimilarity (TPD) metrics; the City Block metric suggests control sub-plot *10d* has experienced rapid change and that *3d* has experienced intermediate rates of change with the communities of sub-plots *13d*, *17a* and *17d* all appearing to be significantly more stable.

The TPD metric suggests a similarly unexpected scenario in which the communities of sub-plots *13a* and *13d* have experience elevated rates of change noticeably higher than that of *10d* (this sub-plot having a more moderate value of average change in community structure).

This lack of agreement over the rates of change experienced by the control sub-plot communities undermines our confidence in the results of the City Block and TPD metric analyses.

The community of Sub-plot *10d* was included as a control for the DCA and dissimilarity metrics because of the high level of confidence we have that it has undergone some of the most dramatic changes in botanical composition of all the Park Grass communities. *10d* has received a combined fertilizer application of N, P, Na, and Mg since 1856 with the nitrogen applied in the form ammonium sulphate at 96kg hectare^{-1}. As already mentioned, this form of nitrogen application has had massive effects on the community structure of this sub-plot. Moreover, the withholding of liming applications otherwise received by sub-plots *10-a*, *b* and *c*, has served to greatly acidify the soil to pH 3.7 - 3.8 at depths of up to 23cm and to pH 4.1 - 4.4 at depths of 23 to 46cm (Thurston *et al.*, 1976). The fertilizers applied to sub-plot *10d* originally included potassium. However, the omission of potassium from 1862 onwards resulted in a considerable increase in *Alopecurus pratensis*, *Agrostis tenuis*

and *Festuca rubra* over the subsequent 15 years (Thurston *et al.*, 1976). Dramatic changes in species composition continued with the increase in *Anthoxanthum odoratum* between 1948 and 1973 to the point where it is now the dominant species. The choice of *3d* as a control sub-plot exhibiting extreme community stability is based on similarly compelling evidence.

Consequently, one might find it difficult to accept that the results of the City Block and TPD metrics accurately depict changes in community compositions on the sub-plots considered. Their results are contradictory to both our own expectations, the results of the Detrended Correspondence Analyses and the Mean Character Difference metric analyses. It can only be suggested that the City Block and TPD metrics are unsuited to our particular composition data sets, perhaps due to sensitivity to large outlying values, or to relative species abundances as was found with the Bray-Curtis measure when investigated by Williams *et al.*, (1973). Consequently, further discussion is focussed on the results of the MCD metric analysis.

With reference to the MCD bar graph of Figure 2.4, an MCD value of ~1.5 (the average change between chronologically consecutive communities on sub-plot *3d*) appears to be indicative of a stable community experiencing minimal change from one time point to the next. A value of ~6.8 on the other hand (the average change between chronologically consecutive communities on sub-plot *10d*), would be suggestive of a community that has experienced considerable and rapid change.

With comparison to these benchmark values, the average MCD value of 4.8 for the sub-plot *10b* community appears to suggest significant change to have occurred over the 138 year time frame considered.

Similarly, the community of sub-plot *16d*, with an average MCD value of 2.8, appears to have experienced an elevated rate change, though not to the same extent as the communities of sub-plots *10b* and *10d*.

Sub-plots *13a* and *13d*, with average MCD values of 2.2 and 2.1 respectively, appear to have experienced more intermediate rates of change in species composition whilst, finally, sub-plots *17a* and *17d* have average MCD values very close to that of sub-plot *3d*, suggesting stability in their community structures over the course of the PGE. However, as with the interpretation of the DCA results, emphasis needs to be placed on the rates of change experienced by each sub-plot over more recent years rather than over the course of the PGE as a whole.

The line graph of Figure 2.7 illustrates the change in each of the communities considered, as measured by MCD metric, with each data point reflecting the difference between the communities of a single sub-plot across two points in time. For example, a data point for sub-plot *10b* at year 2000 represents the difference between the *10b* community in 1974 and the same community in 2000.

Consequently, the final data points of Figure 2.7, relating to change in community structures on each sub-plot between 1973/4/5 and 2000, clearly show sub-plots *10b* and *16d* have experienced significantly higher rates of change than the four remaining sub-plots under consideration.

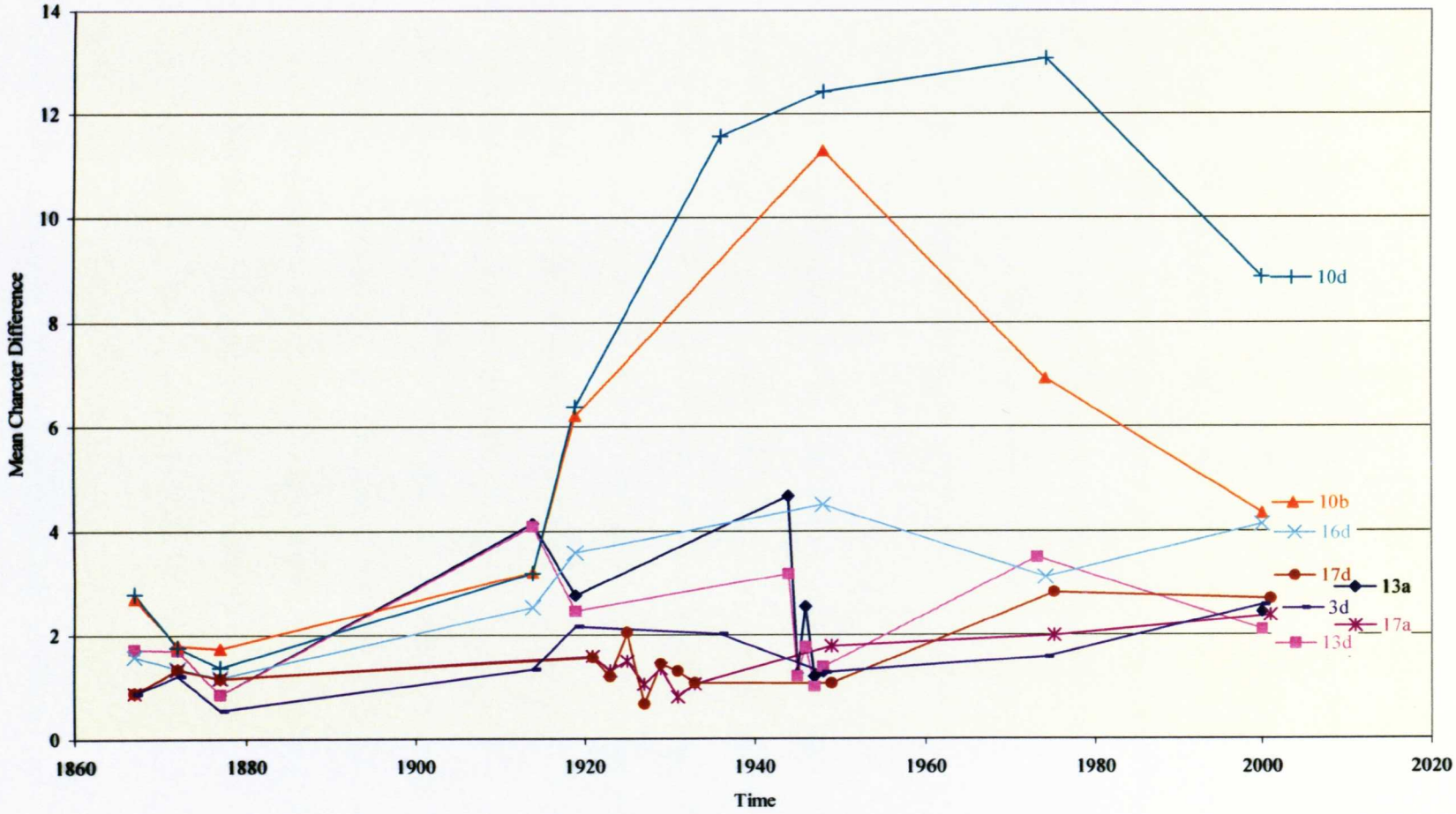


Figure 2.7: Change in Mean Character Difference values on all plots between 1862 and 2000. Each data point represents the difference between the community of a single sub-plot at the time point indicated and the same community from the previous time point.

The MCD values for the sub-plot *10b* and *16d* communities are 4.34 and 4.13 respectively. If we compare these figures with the *average* MCD value of sub-plot *3d*, they suggest a rate of change in community structure on these two sub-plots over the last time frame to have been approximately 3 times that expected from a stable community within the Park Grass meadow. Even if we consider the *actual* MCD value from the last time frame for sub-plot *3d*, which at 2.57 is higher than the average value for this control sub-plot, we still find that the communities of sub-plots *10b* and *16d* have changed almost twice as much, whereas the remaining sub-plots have changed either less or to approximately the same degree as sub-plot *3d*, (MCD values referring to the last time frame for sub-plots *17a*, *17d*, *13a*, and *13d* range from 2.1 to 2.68).

If we consider the MCD values for the 1946/8/9 – 1973/4/5 time frames, we can see that control sub-plot *10d* and sub-plot *10b* both experienced far greater changes in community structure over this period than over the final time frame. Furthermore, sub-plot *10b* experienced an even more dramatic change in community structure over the 1919 to 1948 time period. In contrast, sub-plot *16d* has a lower MCD value for the 1948 - 1974 period than for of the 1974-2000 period.

The remaining sub-plots, *17a*, *17d*, *13a* and *13d* all appear to have experienced relative stability in community structure since 1919/21, although sub-plot *13a* experienced a short period of increased change over the 1919-1944 period. However, it is unlikely that this 25 year period of change, which took place over 60 years ago, will still be detectable in the contemporary ratio of clonal to sexually reproduced genotypes.

Sub-plot *13d* also experienced a moderate increase in rate of change over 31 years ago during the 1948-1973 time frame, but for similar reasoning as attributed to sub-plot

13d, and since the increase in rate of change was far less dramatic, it again seems unlikely that this will be reflected in the structure of the current *F. rubra* population of this sub-plot.

With emphasis placed on observed rates of change over more recent years, the MCD metric analyses suggest that sub-plots *17a*, *17d*, *13a* and *13d* have experienced moderate to low rates of change in community structure. However, sub-plot *10b* appears to have undergone significant and rapid changes in floristic composition as has sub-plot *16d*, though not quite to such a dramatic degree.

Conclusions

There is strong correlation amongst results from the Detrended Correspondence Analysis and the Mean Character Difference analysis. Both analytical methods suggest rates of change in community structure on the control sub-plots in line with predictions based on their widely contrasting histories of fertilizer treatment; rate of change is shown to have been low on sub-plot *3d* and high on *10d*.

The observed agreement of these analytical techniques, not only with one another but also with the predictions of control sub-plot rates of change, suggests a high level of confidence can be placed in their results. Conversely, the lack of congruence in the findings of both the City Block and Proportional Dissimilarity metrics with those of any other analysis technique, or with our own predictions, has led to the conclusion that these two metrics are not well suited to this particular data set and their results should not be considered further.

Both the DCA and the MCD analysis suggest sub-plot *16d* has experienced elevated rates of community change over the course of the PGE, though the rate suggested by the MCD analyses is not as high as suggested by the DCA. Notably, the observed rate of change on sub-plot *16d* is similarly high over recent decades and consequently one would expect this to be reflected in the ratio of clonal to sexually reproduced genotypes.

The communities of sub-plots *17a*, *17d*, *13a* and *13d* are shown via both DCA and MCD analyses to have experienced far more moderate rates of community, and hence environmental, change. The MCD metric analysis suggests that, over the past two time frames (~1948 to ~1975 and ~1975 to 2000) these four sub-plots have experienced community change comparable to that of the control sub-plot *3d*. Only sub-plot *13a* shows any significant yet temporary increase in rate, after which the community returned to relative stability.

However, the DCA suggests these four sub-plots have experienced change that is more intermediate to those of the control sub-plots. Consequently, one can only conclude from these two sets of results that whilst environmental change is likely to have taken place on these four sub-plots, it is unlikely to have occurred at a significantly elevated rate.

As with sub-plot *16d*, the rate of change on sub-plot *10b* is estimated to be high using one analysis technique (MCD), yet only moderate using the other (DCA). Hence, one might argue that the true rate of environmental change experienced by populations on sub-plot *10b* is likely to be somewhere between intermediate and high.

From the results of this Chapter, the *Festuca rubra* populations of sub-plots *10b*, *16d*, *13a*, *13d*, *17a* and *17d* were chosen as the focus of this study. Microsatellite markers have been employed to distinguish between clonally and sexually derived genotypes

of individuals in order to determine the mode of reproduction selected for in each of these populations. However, the discovery that *F. rubra* is represented by two sub-species on Park Grass posed problems related to the analysis of microsatellite data. Consequently, in the next Chapter, molecular methods that were employed with the aim of distinguishing between the *F. rubra* sub-species from the different sub-plots prior to microsatellite analyses are described.

Chapter 3

Development of a potential diagnostic marker within the ITS1 region of the nuclear ribosomal DNA for distinguishing *F. rubra* ssp. *rubra* and ssp. *commutata*

Introduction

The unexpected discovery of two *Festuca rubra* sub-species coexisting on the Park Grass meadow initially posed complications regarding the analysis of associated microsatellite genotype data. As described in Chapter 1, these two sub-species from the polyploid red fescue lineage differ in ploidy level and hence are likely to represent reproductively isolated units, since any hybrid offspring would theoretically carry unbalanced chromosome numbers, rendering them inviable. Consequently it is necessary to distinguish populations of one sub-species, from those of the other before interpreting microsatellite data.

The simplest method of determining the sub-species of each sample would have been via morphological analysis, prior to the extraction of DNA and subsequent amplification and scoring of microsatellite loci. Unfortunately, discovery of the existence of two *F. rubra* sub-species on Park Grass didn't take place until all sample collections, DNA extractions and amplifications had been carried out. The two sub-species are extremely similar morphologically, with the principal distinguishing characteristic being the presence of rhizomes in ssp. *rubra* individuals.

Having collected samples from the field and extracted DNA from each, the remaining leaf material was unfortunately insufficient in quantity to allow for further morphological analysis. Consequently, sub-species identification of each sampled individual was only feasible by analysis of extracted DNA, and for this purpose the Internal Transcribed Spacer 1 region (ITS1) of the nuclear genome was considered for sequence analysis.

The ITS1 is a part of a multicopy nuclear gene encoding subunits of the ribosomal RNA. In higher plants the ITS1 region is flanked by the 18s and 5.8s genes. A second

Internal Transcribed Spacer also exists, (ITS2), which is flanked by the 5.8s and 26s genes (Wendel *et al.*, 1995). The ribosomal DNA (rDNA) genes with the two internal transcribed spacers and a further external transcribed spacer, located upstream of the 18s gene, constitute the rDNA repeat unit. These repeat units are present in paralogous copies in plant nuclear genomes, usually numbering in their thousands (Gaut *et al.*, 2000; Hillis and Moritz, 1990), and are separated from one another by non-transcribed spacers (see Figure 3.1).

The rDNA genes have been widely used for phylogenetic purposes at the family and higher taxa levels (Hamby and Zimmer, 1988). However, in contrast to these coding rDNA genes, the non-coding ITS1 and ITS2 are relatively fast evolving (Ruggiero and Procaccini, 2004; Wendel *et al.*, 1995). Nonetheless, evolution of the internal transcribed spacers is still governed by selective constraints; the spacers contain signals necessary for processing of the rRNA transcript (Hillis and Dixon, 1991) and they both have stable and compact secondary structures facilitating their functional roles (Reed *et al.*, 2000).

The internal transcribed spacers have typically proven phylogenetically informative at the interspecific level (Gaut *et al.*, 2000; Jobst *et al.*, 1998) and have also served as population level markers suitable for investigations into hybridisation and in the identification of distinct strains of organisms such as within the species *Sceloporus grammicus* (Sites and Davis, 1989) and *Vicia faba* (Rogers *et al.*, 1986).

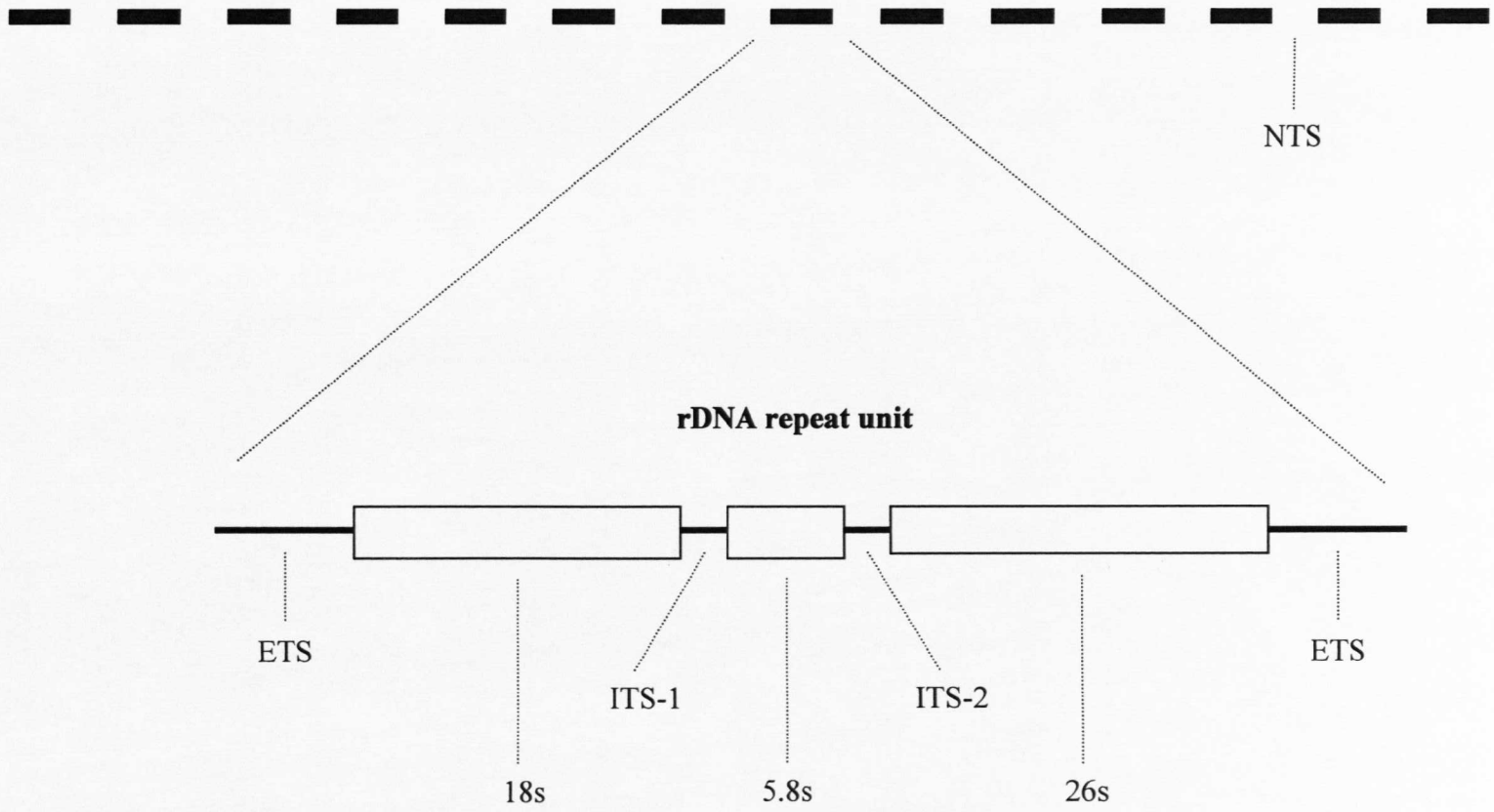


Figure 3.1: Diagrammatic representation of ribomsomal DNA repeat structure in angiosperms (though the 26s gene may be represented by homologous genes in other species). ETS and NTS refer to external transcribed spacers and non-transcribed spacers respectively.

In some species, rDNA arrays may be found at more than one chromosomal locus (Jorgensen and Cluster, 1988; Wendel *et al.*, 1995) and intraindividual copy numbers may vary as much as four fold (Long and David, 1980). Given the number of rDNA repeat units within a single individual and the potential for these to be spread across several loci, one might expect there to be high intraindividual ITS sequence diversity. Indeed, polymorphism of the ITS region at the intraindividual level is a recognised feature amongst both plants and animals (Buckler and Holtsford, 1996; Campbell *et al.*, 1997; Suh *et al.*, 1993). For example the marine angiosperm *Halophila stipulaceae* exhibits ITS polymorphism ranging from the interpopulation level through to the intraindividual level (Ruggiero and Procaccini, 2004).

A further source of ITS sequence variation exists in the form of pseudogenes and in fact most of the ITS variability described previously in *Halophila stipulaceae* was found to represent pseudogenic sequences (Ruggiero and Procaccini, 2004). Since ITS pseudogenes have also been identified in species of the genus *Lolium* (Gaut *et al.*, 2000), close relatives of *Festuca* species, it is good practice to exercise the simple procedures that exist for screening for pseudogenes within arrays of ITS sequences before determining levels of sequence heterogeneity and drawing further conclusions. Factors such as evolutionary rate, free energy of secondary folding structure and reduced G-C contents resulting from elevated levels of methylation mutations are all used as identifiers of pseudogenic sequences (Ruggiero and Procaccini, 2004), and the G-C contents of Angiosperm functional ITS1 and ITS2 sequences are normally found to lie between 50 and 75% (Baldwin *et al.*, 1995).

Nonetheless, whilst polymorphism of ITS regions both within and amongst individuals does occur, in most species the majority of these copies are identical (Wendel *et al.*, 1995). There are several processes to which this observed

homogeneity in ITS copies may be attributed, including unequal crossing-over during meiosis and gene conversion, these being thought of as most significant (reviewed by Hillis & Dixon, 1991). Alternatively, intraindividual homogenisation of rDNA repeats may occur as a result of 'rDNA locus loss' (Skorupska and Palmer), resulting in the complete deletion of what may have been a heterogeneous ITS morph from a lineage. Collectively these processes, which act to homogenise the ITS copies within individuals and indeed populations, are referred to as concerted evolution (Hillis *et al.*, 1991; Ruggiero and Procaccini, 2004; Wendel *et al.*, 1995).

With the homogenising effects of concerted evolution in mind, one might wonder what circumstances facilitate the persistence of ITS polymorphism within species and indeed individuals. The simplest explanation would be a lack of recombination, prohibiting unequal crossing over and its associated homogenising effects. Using this reasoning, we might conversely expect the ongoing effects of sexual recombination within species to be reflected in low levels of rDNA repeat unit polymorphism, yet Weeks & Simpson, (Weeks and Simpson), list several studies on both hybrid and non-hybrid plants in which this is quite the opposite. Similarly, in the absence of recombination, gene conversion may confound our expectations of higher intraspecific repeat unit polymorphism (Hillis *et al.*, 1991).

Possibly of greater relevance to the sub-species studied here are the consequences of polyploidy on ITS sequence diversity. Species and races of higher ploidies often exhibit greater diversity than lower ploidies (Gaut *et al.*, 2000). This is likely to be due to polyploid species possessing greater chromosome complements than diploid species and consequently larger numbers of rDNA loci and associated ITS variants. Furthermore, the combination of divergent ITS variants from different progenitor

species within the single genome of an allopolyploid individual is also likely to increase ITS variant numbers relative to individuals of autopolyploid species.

It is not known whether ssp. *rubra* and ssp. *commutata* are auto- or allopolyploid. However, should they be allopolyploid, as has been suggested of species of the *Festuca-Lolium* complex in general (Gaut *et al.*, 2000), and proven for the hexaploid *Festuca arundinaceae* (Berg *et al.*, 1979), then it is likely this would be reflected in elevated levels of ITS sequence diversity. What is more, the high ploidies of the two sub-species studied here is, as explained above, likely to further increase levels of intraindividual ITS sequence heterogeneity. These factors might be seen to reduce our chances of finding a sub-species-specific molecular marker based in the ITS region, since greater intraindividual diversity may reflect a lower likelihood of identifying sequence variants specific to one sub-species or the other because of incomplete lineage sorting.

However, the genus *Festuca* contains species ranging from diploid, (2n = 14), to decaploid, (10n = 70). Coupled with the broad geographic distribution of *Festuca* species, this factor might suggest the genus is older than the closely related genus *Lolium* whose species are reportedly of uniform ploidy (Malik and Thomas, 1967). The genus *Lolium* is estimated to have diverged from the broad-leaved fescues approximately 2 million years ago (MYA) and differentiated into contemporary species about 1 MYA (Charmet *et al.*, 1997). Whilst the fine-leaved fescues, which include *F. rubra*, are also of relatively recent evolutionary origin, having diverged from the broad-leaved fescues and subsequently radiated by about 9 MYA (Charmet *et al.*, 1997), the comparatively greater depth to their evolutionary history may have resulted in complete lineage sorting amongst their respective ITS sequences, allowing for distinction of the two sub-species considered. It should be noted though, that

complete lineage sorting may be confounded by hybridisation and introgression, or by recurrent polyploidisation events, such as those described in *Tragapogon* species (Soltis *et al.*, 1995).

Nontheless, an alignment by Hsiao, (Hsiao *et al.*, 1995), of the entire ITS region from 26 species, 25 of which were from the subfamily Pooideae, revealed 49.4% of sites in the ITS1 region to be informative. Whilst this alignment did not include a *Festuca rubra* sequence, both *Festuca mairie* and *Lolium perenne,* which are from the same tribe, *were* represented. This suggests a reasonable likelihood of identifying informative sites at the species and, potentially, at the sub-species levels.

Based on the information outlined above, it was decided that analysis of ITS1 region sequences from individuals known to represent each of the two sub-species might allow for identification of a marker capable of distinguishing them. We would expect that, given sufficient time since divergence and the assumption that introgression has not occurred, distinction of the sub-species via ITS sequence variation will be possible. Furthermore, if as already postulated, the sub-species are allopolyploids, then gene conversion may have taken place toward one of the parental rDNA repeat types in either sub-species (Buckler and Holtsford, 1996), increasing the potential for their distinction via this targeted region.

Methods

Collection of samples of known sub-species: Having gained special permission from the Rothamsted Research Committee, a limited number of samples were collected as whole plants from various points across the Park Grass meadow early in the growing

season. This allowed not only for DNA extraction from leaf material, but also for further growth of the individuals in the greenhouse so that they might reach maturity. Consequently, any characteristics relevant to morphological identification of the sub-species which were not yet present due to immaturity of the collected material were allowed to develop. In this way our preliminary sub-species identifications, based on morphological assessments, could be confirmed or amended at a later date. Hence, our subsequent assignment of the sequences as representing one sub-species or the other could be carried out with confidence.

DNA extraction and standardisation: DNA was extracted from samples in batches, each of which included a negative control. A CTAB plant DNA extraction protocol (Porebski *et al.*) suitable for leaf material with high levels of polysaccharides and polyphenols was employed using approximately 20mg of leaf material ground in liquid nitrogen. After extraction DNA was eluted in 100 µl of ddH_2O and stored at -20°C.

Before amplification of the target region, aliquots of each extraction were quantified for DNA concentration on a FLUOstar Optima Spectrophotometer using calf thymus DNA as a standard. The extraction aliquots were then standardised for DNA concentration using a Beckman Coulter 2000 laboratory automatic workstation to 10ng/µl.

Primer design: A primer pair shown to amplify 610bp from the ITS and 5.8s regions (ITS1-5.8s-ITS2) in various *Festuca* species (Torrecilla *et al.*, 2003) was tested on *Festuca rubra* samples but revealed inconsistent amplification in individuals collected from the PGE. Consequently, homologous sequences from nine *Festuca* species and

three *F. rubra* sub-species (listed below in Table 3.1 with Accession numbers) were downloaded from GenBank and aligned using ClustalX (Thompson *et al.*, 1997). With respect to the pairwise alignment parameters the 'gap opening' and 'gap extension' values were set at 10.00 and 0.10 respectively. The same modifications were made to the multiple alignment parameters and the 'delay divergent sequences' setting was changed to 25%. Otherwise all default alignment settings suggested by the ClustalX program were accepted.

The sequence alignment was submitted to the 'Primer3' software package (Rozen and Skaletsky, 2000) online, to identify suitable primers for fragment amplification in *Festuca rubra.* Two primer pairs were designed amplifying fragments within the ITS1 region. These were named ITS1-A-forward and reverse and ITS1-B-forward and reverse amplifying target regions of 196bp and 250bp respectively. The primer sequences are given below in Table 3.2.

The ability of the two primer pairs to consistently amplify their target fragments was then tested using a sample set consisting of sixteen individuals comprised of eight *F. r. rubra* genotypes and eight *F. r. commutata* genotypes. The sub-species status of these sixteen samples had been previously confirmed via analysis of morphological characteristics, including the presence/absence of rhizomes, as described by Hubbard, (1984).

***Table 3.1*:** Sequences used in the ITS region alignment for the design of ITS1 primer pairs.

Species	GenBank Accession No.
F. quadriflora	AF519980
F. dimorpha	AF519975
F. pulchella jurana	AF519978
F. pulchella pulchella	AF519976
F. pseudeskia	AF519977
F. scariosa	AF519979
F. spectabilis	AF519982
F. arundinaceae	AF519983
F. aragonesis	AF519981
F. rubra rubra	AF147176
F. rubra rubra	AF147143
F. rubra rubra	AF147141
F. rubra fallax	AF147162
F. rubra fallax	AF147164
F. rubra fallax	AF147148
F. rubra littoralis	AF147163
F. rubra littoralis	AF147145
F. rubra littoralis	AF147150

Table 3.2: Sequences and relevant details of the two primers pairs designed for amplification of fragments within the ITS1 region.

Primer name	Sequence	*Position	Fragment name	Fragment size
ITS1-A-forward	GAA GGC GTC AAG GAA CAC TG	138	ITS1-A	196bp
ITS1-A-reverse	TTG CGT TCA AAG ACT CGA TG	333		
ITS1-B-forward	TGG TGT GAA TTG CAG AAT CC	287	ITS1-B	250bp
ITS1-B-reverse	CGG ATG CAC TGC GTT GTT TAG TA	536		

* = In relation to the consensus sequence generated via the alignment of homologous sequences from various *Festuca* species and sub-species, listed in Table 3.1.

PCR Amplification: Amplification of ITS1-A fragments was performed in 25µl volumes using a 1x PCR Reaction Buffer (ABgene), 1.5mM magnesium chloride, 2.0µM of each primer, 0.75 U Taq polymerase (ABgene), 0.4mM dNTPs (ABgene) and 20ng of extracted nucleic acids. A hot-start PCR cycle was performed on a Techne Touchgene Gradient PCR machine under the following conditions: an initial denaturation was carried out at 94°C for two minutes followed by 35 cycles of 94°C for one minute, 64°C for 30 seconds and 72°C for 30 seconds. A final extension step was performed at 72°C for ten minutes.

Amplification of the ITS1-B fragment was optimised to run using the same reagent concentrations given above. However, the cycling parameters were slightly altered and the annealing temperature increased to 65°C. Nonetheless, amplification from *F. rubra* individuals using this primer pair proved extremely inconsistent. Hence, the ITS1-B primer pair was deemed unsuitable for further investigations and the remainder of this methods section consequently refers to investigations carried out using the ITS1-A primer pair.

All sets of individual PCRs were run with the inclusion of a negative control to screen for any possible contamination.

Cloning and Sequencing: Cloning of amplified products allowed us to determine whether prior success in direct sequencing resulted from intra-individual homogeneity in ITS sequence repeats.

A total of 16 individuals were sequenced directly from PCR products. Eight of these represented ssp. *rubra* whilst eight represented ssp. *commutata*, as identified via morphological analyses. After repeated efforts, PCR products were successfully cloned from 11 of these 16 individuals using a pGEM®-T Easy Vector kit (Promega).

Prior to cloning, the PCR products and negative controls were run alongside a quantification ladder (Hyperladder IV; Bioline) on 1.2% agarose gels. This allowed for estimation of amplified product concentration and optimisation of the insert: vector ratio in the ligation reactions which, as with the subsequent transformation reactions, were carried out in line with the manufacturers' protocol.

Preliminary identification of positive-insert plasmid colonies was carried out using the blue-white screening procedure as described, again, in the manufacturer's protocol. Cloning of an insert in the pGEM®-T Easy Vector interrupts the coding sequence of β-galactosidase and as a consequence the colonies of positive-insert plasmids are white as opposed to blue. However, final confirmation of positive insertion was gained through amplification of cloned products from white colonies using the primers designed in this study and colony 'picks' as DNA templates. With the amplified products run on agarose gel, visualization of fragments of the expected length confirmed that cloning of an appropriate insert had indeed taken place whereas absence of a band informed us that cloning had not been successful.

Prior to sequencing, the PCR products were subsequently purified using QIAquick PCR purification kits (Qiagen). Sequencing was carried out on an ABI 377 automated capillary sequencing facility (Applied Biosystems) using BigDye terminator mix 1.1 (Applied Biosystems) according to the manufacturers' recommended protocol. Each cloned fragment was sequenced in both directions.

Screening for pseudogenes:

Calculations of the percentage G-C contents for all sequences obtained in this study were made using BioEdit software (Hall, 1999). This allowed for preliminary

identification of any pseudogenic sequences such that further analyses could be carried out if necessary.

Sequence Analysis: The 196bp sequences obtained for the ITS1-A region were manually edited using Sequence Navigator version 1.0.1 (PE Applied Biosystems). Using complementary forward and reverse sequences, consensus sequences were generated for each cloned insert. Primer sequences were trimmed and nucleotide ambiguities were checked by eye.

Alignments were performed on the sequences generated in this study using the program ClustalX (Thompson *et al.*, 1997) under the same alignment parameters as described above.

Statistical parsimony networks linking sequences variants, or haplotypes, were then generated using TCS 1.18 software package (Clement *et al.*, 2000).

Results

Eight individuals representing ssp. *commutata* were sequenced directly from amplified products of the ITS1 region. In contrast, direct sequencing of PCR products from eight ssp. *rubra* individuals failed to generate readable sequences.

Previous studies have taken success in direct sequencing of PCR products as evidence of intraindividual sequence homogeneity (Hunter *et al.*, 1997; Medina *et al.*, 1999). Indeed a study by Hsiao *et al.*, (Hsiao *et al.*, 1995), investigating the phylogenetic relationships amongst members of the Pooideae (Poaceae), was based solely on the

direct sequencing of amplified ITS region products from individual representatives of each of the 25 species considered.

To determine whether successful direct sequencing could be taken as indication of intraindividual sequence homogeneity within our samples, PCR products were cloned and sequenced from a subset of the eight individuals previously sequenced directly from PCR. This revealed that, of those cloned individuals, successful direct sequencing was not indicative of within-individual sequence homogeneity since multiple ITS1 variant sequences were obtained from each individual. Consequently, all further sequencing was carried out on cloned amplified products.

A total of 11 individuals (eight ssp. *rubra* and three ssp. *commutata*) were successfully cloned and a further 40 sequences generated from the 196bp ITS1-A fragment, representing between one and four variants per individual.

Sequence variation was observed in the form of three separate insertion/deletion events present in a total of eight sequences, and six transitions/transversions which were present in a total of 13 sequences (see alignment of all sequences generated in this study in Appendix 4). Maximum divergence between sequences generated here was 3.17%, whilst that between sequences generated from different sub-species was just 1.27%.

Of the 11 individuals that were cloned and sequenced from, only four were found to reveal a single ITS sequence variant, or haplotype, with two of these individuals being represented by a single sequenced clone.

Two sequence alignments were generated; an alignment of full-length sequences (primer sequences removed), and an alignment of shorter sequences allowing for the inclusion of those generated directly from PCR and two further cloned sequences, each of which failed to provide reliable reads for their entire length. From these

alignments, two statistical parsimony networks were produced which are displayed below in Figures 3.2 and 3.3. One network is based on those haplotypes, distinguishable in the full-length alignment, whilst the second is based on the haplotypes of the shorter alignment.

Tables 3.3 and 3.4, below, list the ITS haplotypes represented by each individual investigated in this study. Again, Table 3.3 refers to those haplotypes distinguishable from one another with reference to the full-length sequence alignment, whereas Table 3.4 lists the haplotypes distinguishable with reference to the shorter alignment. Whilst some haplotypes appear to be represented exclusively by individuals of one sub-species or the other, we cannot state this as fact due to the low sample sizes. However, it is apparent that no haplotype is represented by all individuals of one sub-species whilst remaining unrepresented amongst individuals of the other sub-species.

Intragenomic variation in sequence composition of the ITS1 region amongst ssp. *rubra* and ssp. *commutata* individuals appears to be negligible and, as a consequence, the haplotypes prove uninformative in distinguishing the sub-species.

Nonetheless, the networks displayed in Figures 3.2 and 3.3 do reveal that the most frequently encountered haplotype, haplotype 4, is present amongst individuals of both ssp. *rubra* and ssp. *commutata*. What is more, haplotype 4 is central to both networks and has a greater number of connections than any of the surrounding haplotypes. Consequently, this suggests haplotype 4 is older and potentially ancestral to the peripheral haplotypes of the two networks.

There also appears to be greater diversity of haplotypes exhibited amongst individuals of ssp. *rubra* than ssp. *commutata*, yet there is no evidence of structuring or sub-branching of haplotypes between the sub-species. This may be due to the subspecies being represented by unequal sample sizes.

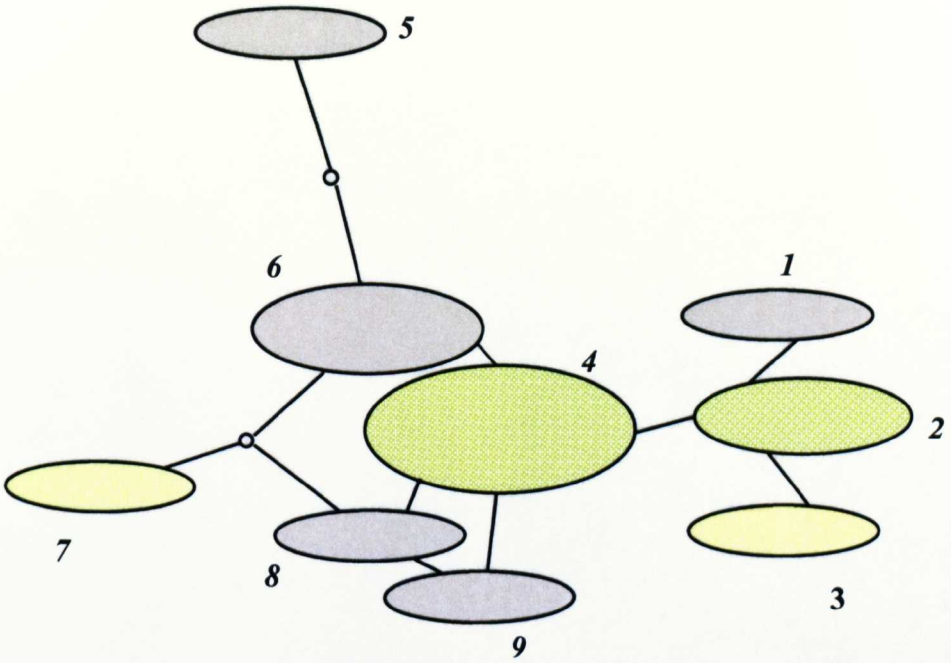
***Table 3.3*:** Haplotypes of ITS sequence variants generated in this study based on full-length sequence alignment

INDIVIDUAL	SUB-SPECIES	TOTAL NO. OF HAPLOTYPES	HAPLOTYPES REPRESENTED
Frr1	ssp. *rubra*	2	2, 4
Frr2	ssp. *rubra*	2	6
Frr3	ssp. *rubra*	1	4
Frr4	ssp. *rubra*	4	5, 6, 9
Frr5	ssp. *rubra*	3	1, 2, 6
Frr6	ssp. *rubra*	1	6
Frr7	ssp. *rubra*	2	4, 8
Frr8	ssp. *rubra*	1	4
Frc1	ssp. *commutata*	3	2, 4, 7
Frc2	ssp. *commutata*	1	3
Frc3	ssp. *commutata*	1	4

Table 3.4: Haplotypes of ITS sequence variants generated in this study based on shorter-length sequence alignment

INDIVIDUAL	SUB-SPECIES	TOTAL NO. OF HAPLOTYPES	HAPLOTYPES REPRESENTED
Frr1	ssp. *rubra*	2	2, 4
Frr2	ssp. *rubra*	2	2, 4
Frr3	ssp. *rubra*	1	4
Frr4	ssp. *rubra*	4	4, 5, 6, 9
Frr5	ssp. *rubra*	3	1, 2, 4
Frr6	ssp. *rubra*	1	4
Frr7	ssp. *rubra*	2	4, 8
Frr8	ssp. *rubra*	1	4
Frc1	ssp. *commutata*	3	2, 4, 7
Frc2	ssp. *commutata*	1	3
Frc3	ssp. *commutata*	1	4
Frc4 to 11*	ssp. *commutata*	1	4

N.B: * indicates sequences generated directly from PCR products, without cloning.

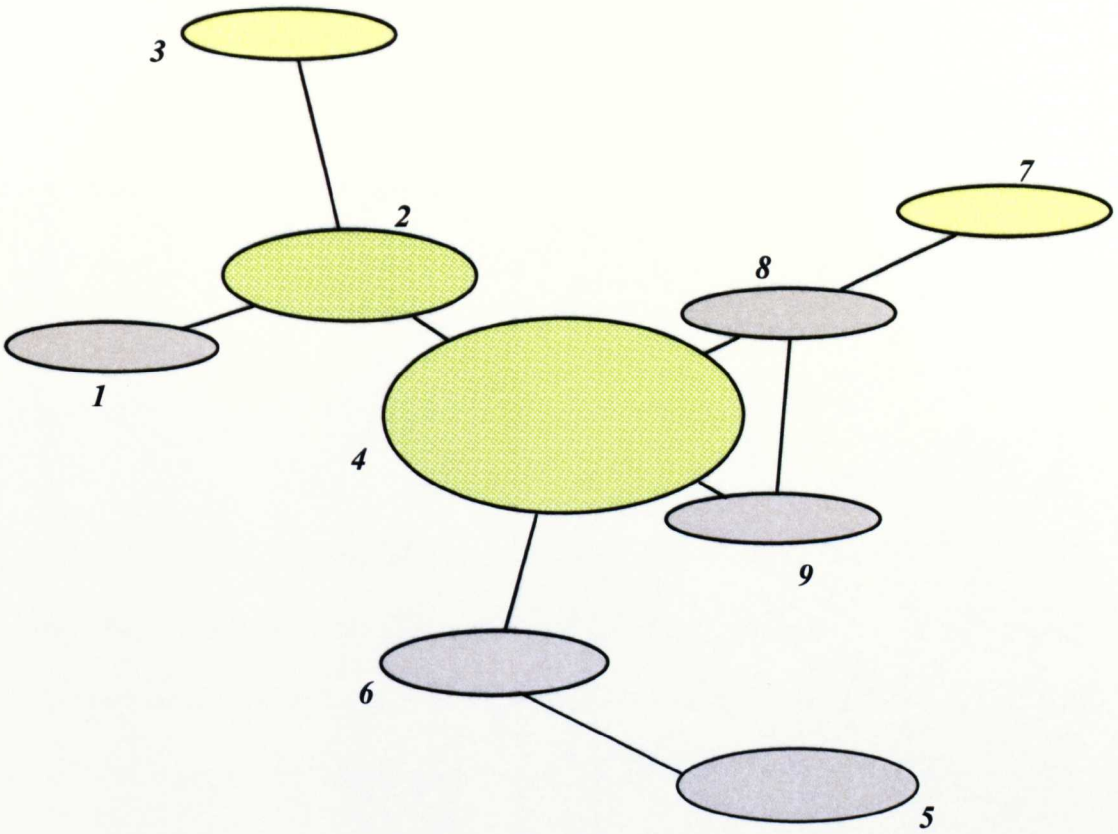


Haplotype represented by individuals of ssp. *rubra*.

Haplotype represented by individuals of ssp. *commutata*.

Haplotype represented by individuals of both ssp. *rubra* and ssp. *commutata*.

***Figure 3.2*:** Network of 9 different ITS1 haplotypes of *Festuca rubra* ssp. *rubra* and ssp. *commutata* inferred from the full-length sequence alignment of 158 bp. Haplotypes are represented as ovals with each haplotypes' number indicated alongside. Size of ovals corresponds to the number of individuals exhibiting the haplotype represented. Branches between haplotypes denote single nucleotide differences, with small circles along the branches representing further single nucleotides differences.



Haplotype represented by individuals of ssp. *rubra*

Haplotype represented by individuals of ssp. *commutata.*

Haplotype represented by individuals of both ssp. *rubra* and ssp. *commutata.*

***Figure 3.3*:** Network of 9 different ITS1 haplotypes of *Festuca rubra* ssp. *rubra* and ssp. *commutata* inferred from the shorter sequence alignment of 145bp. Haplotypes are represented as ovals with each haplotypes' number indicated alongside. Size of ovals corresponds to the number of individuals exhibiting the haplotype represented. Branches between haplotypes denote single nucleotide differences.

The G-C contents of the 48 ITS1 fragment sequences generated in this study are all very similar to one another, ranging from 53.24% to 56.41%. These values are within the expected G-C content range of Angiosperms (Baldwin *et al.*, 1995) and show no evidence of pseudogenic properties.

Conclusions

At the outset of our sequencing studies into *Festuca rubra* ssp. *rubra* and ssp. *commutata* it was hoped that divergence in their respective ITS variant sequences, or haplotypes, might become apparent allowing for determination of an individual's sub-species status in the absence of morphological information. The date of divergence of the broad-leaved fescues from the fine fescues is estimated to have taken place approximately 9 million years ago (Charmet *et al.*, 1997). Of the subsequent radiations within the fine-leaved fescues that of the *Festuca rubra* aggregate is suggested to have been earliest (Tredway, 1999) and this contention is supported by the maximum parsimony analyses carried out by Gaut *et al.*, (Gaut *et al.*, 2000), in their phylogenetic study of the *Festuca-Lolium* complex.

Consequently, it was expected that the evolutionary history of the *F. rubra* aggregate would provide sufficient time for concerted evolution and complete lineage sorting to take effect, assuming hybridisation to have not occurred. The highly polyploid nature of the *F. rubra* complex and the associated implications on reproductive isolation of the two sub-species considered here serve to emphasise these expectations. However, relative to diploid species, the large gene-population size consequential of polyploidy may have elevated ITS sequence variability at intraindividual through to intraspecific

levels, increasing the length of time required for concerted evolution and lineage sorting to reach completion. Nonetheless, the maximum sequence divergence amongst haplotypes of 3.17% is extremely low considering time since divergence of the fine fescues. What is more, maximum divergence between haplotypes of the two sub-species is even lower at 1.27%. These values are reflected in the patterns of the haplotype networks which are characteristic of a single reproductive unit or at least very recent, incomplete divergence of species/sub-species. Indeed, concerted evolution of rDNA repeats in subspecies *rubra* and *commutata* may still be ongoing, accounting for the observed sharing of haplotypes. A similar suggestion was provided by Brouat *et al.*, (Brouat *et al.*) upon discovery of limited ITS and intergenic marker differentiation amongst species and sub-species of the genus *Leonardoxa.*

Alternatively, the polyploidisation events that generated sub-species *rubra* and *commutata*, estimated to have taken place ~1 million years ago (Charmet *et al.*, 1997), may have been the first of many. Multiple origins have been described for numerous other polyploid species including members of the genus *Tragopogon* (Soltis *et al.*, 1995) and *Draba* (Brochmann *et al.*, 1992). Such recurrent generation of individuals might also account for the observed incomplete concerted evolution.

As mentioned earlier in this chapter, ssp. *rubra* and ssp. *commutata* are expected to be reproductively isolated from one another due to their differing ploidies and the theoretical inviability of any hybrid offspring. Almost all models of population establishment and maintenance with respect to polyploids make the assumption that intercytotype hybrids are lethal. However, as is often the case in biology, our expectations can prove restrictive compared to reality; reproduction by inter-cytotype hybrids has in fact been recorded, either through backcrossing with parental cytotypes or through recombination with other hybrid individuals (Husband, 2003). The triploid

bridge, for example, refers to a mechanism in which the unreduced gametes of a triploid hybrid, (which itself may have been generated through the union of n = 2x and n = x gametes), backcross with the reduced gametes of a diploid parental cytotype. The resulting union of n = 3x and n = x gametes, or other combinations of reduced and unreduced gametes, may result in tetraploid individuals in the F2 generation. Indeed, Soreng & Davis, (Soreng and Davis), describe hybridisation as one of the main evolutionary processes operating amongst Poeae grasses. Furthermore, owing to the relatively recent origin of the polyploid red fescues (Charmet *et al.*, 1997), Catalan, (Catalan *et al.*), suggests members of this lineage are more likely to experience recurrent interspecific introgression than species holding more distant relationships with one another.

Consequently, hybridisation and introgression may well have had an ongoing and confounding influence on lineage sorting of ITS1 region haplotypes, resulting in the observed sharing of haplotypes amongst the two sub-species considered here. Alternatively, complete lineage sorting may have been achieved at some point in the past; only to be disrupted by more recent hybridisation events.

Whilst pseudogenes that have only recently lost function, and consequently accumulated insufficient mutations to significantly change their GC contents, may exist within our set of haplotypes, the fact that more ancient pseudogenes have not been detected suggests their frequency in the targeted population of *Festuca rubra* is uncommon. Hence, the occurrence of pseudogenes offers an unlikely explanation for the sharing of haplotypes by the two sub-species. However, discriminating between the consequences of shared ancestral polymorphism/incomplete lineage sorting and hybridisation is far more difficult (Wakeley, 1996). Bayesian coalescent approaches have proven useful in this respect with regard to single-copy genes (Vollmer and

Pallumbi, 2002), but there is no framework available for distinguishing between the two processes that can be applied to rDNA repeats (Vollmer and Pallumbi, 2004).

Concerted evolution and lineage sorting of ITS sequences have been assumed to occur amongst of members of the Poaceae due to the detection of unique sequences in most taxa sampled (Hsiao *et al.*, 1995). However, Gaut *et al.*, (Gaut *et al.*), carried out extensive intraspecific samplings of *Festuca* species which revealed polyphyletic and paraphyletic lineages of *F. mairie*, *F. pratensis* and *F. arundinacea*. Consequently, this information might suggest incomplete lineage sorting as a more likely explanation for the shared sequences of sub-species *rubra* and *commutata* than hybridisation and introgression.

The reason behind the success in direct sequencing from the eight *F. r. commutata* individuals but not the *F. r. rubra* individuals, even though intraindividual variation is high in both sub-species, remains unclear. This result could simply represent the preferential amplification of one sequence variant over those remaining. The sequences generated through direct sequencing from the eight ssp. *commutata* individuals were indeed identical which would support this suggestion.

In conclusion, analysis of ITS1 haplotypes from *F. rubra* ssp. *rubra* and ssp. *commutata* individuals has provided no diagnostic information with which to distinguish the sub-species. Similarly, it provides no suggestion as to the state of introgression and hybridisation between the two. Future studies aiming to identify a diagnostic marker or region capable of distinguishing these sub-species should target the ITS repeat unit as a whole, i.e. the ITS1, 5.8s and ITS2. This larger unit is more likely to contain a sufficient number of variable sites and may prove informative at the intraspecific level. Alternatively a fast evolving single copy region could be targeted, such as the chloroplast *trn*L-*trn*F intergenic spacer sequence which has

proved informative at the intraspecific level in other taxa (Brouat *et al.*, 2001; Gielly and Taberlet, 1994).

The implications of these findings on the analysis of microsatellite data are significant. Sub-species of each genotyped individual cannot be unambiguously assigned, and whilst it has not been possible to rule out the occurrence of introgression and hybridisation between the sub-species, the unbalanced chromosome numbers that any hybrid progeny are likely to carry, is nonetheless expected to represent a reproductive isolating mechanism.

In order to identify which, if any, of the populations of this study are represented by a single sub-species, flow cytometric analyses were performed. These also provided a means of determining the state of introgression and hybridisation between the sub-species. The results of these analyses are presented in Chapter 4.

Chapter 4

The ratios of two *Festuca rubra* cytotypes on plots of the PGE and factors facilitating their coexistence

Introduction

The two sub-species, *Festuca rubra* ssp. *rubra* and ssp. *commutata*, represent different cytotypes within the *F. rubra* complex and this fact is reflected in their respective nuclear DNA contents. The hexaploid ssp. *commutata* has a reported nuclear DNA content range of 11.24 to 12.37 picograms (pg), (sub-species mean = 11.91pg) whereas the octaploid ssp. *rubra* ranges from 12.54 to 15.53pg/nucleus (sub-species mean = 14.27pg) (Huff and Palazzo, 1998). Theory dictates that the two cytotypes are likely to be reproductively isolated from one another because of their differing ploidies, since the unbalanced chromosome numbers of any hybrid offspring are expected to render them inviable.

Attempts to identify a molecular genetic marker within the Internal Transcribed Spacer region of the nuclear ribosomal DNA capable of distinguishing these sub-species were not successful. This was due either to recent hybridisation, or lack of evolutionary or sequence divergence (or both). However, the fact that the two sub-species represent different cytotypes within the *F. rubra* complex offers an alternative method of sub-species identification.

Whilst it is not possible to determine ploidy levels of those individuals in this study for which DNA has already been extracted, it is possible to determine the relative frequencies of cytotypes within the contemporary populations from which the genotyped individuals were collected. This would enable the identification of any populations as monomorphic for one cytotype or the other. Such populations could then be analysed without the complications generated by the possible presence of two cytotypes whilst estimates of gene flow amongst any such monocytotypic populations could also be made. Importantly, determination of the relative frequencies and

distribution of cytotypes would also prove valuable in allowing us to infer the evolutionary forces governing establishment and maintenance of any mixed cytotype populations.

The variation in ploidy observed amongst sub-species of the *F. rubra* complex is by no means unusual. Polyploidy is common amongst perennial taxa capable of vegetative reproduction (Stebbins, 1938). Perennials have the advantage over annuals in terms of time and opportunities with which to find mates of the same cytotype, whilst vegetative reproduction facilitates the generation of more local partners with which intracytotype mating can take place (Stebbins, 1950).

The most common mechanism of polyploid formation is through the union of unreduced gametes (Bretagnolle and Thompson, 1995; Ramsey and Schemske, 1998) generated as a consequence of meiotic dysfunction (Bretagnolle and Thompson, 1995). If such a union involves the unreduced gametes of individuals from the same species (2n x 2n = 4n), the resulting offspring are referred to as autopolyploids. However, if the unreduced gametes are contributed by individuals from different species, the resulting hybrid offspring are referred to as allopolyploids.

The terms autopolyploid and allopolyploid were first coined by Kihara and Ono in 1926. Cytotypes of either of these polyploid forms are likely to display novel phenotypic and physiological characteristics (Stebbins, 1950) unexpressed in their progenitor cytotypes. This may be due to relaxed evolutionary constraints on the function of duplicated genes resulting in divergence and neofunctionalisation of phenotypes (Osborn *et al.*, 2003; Wendel, 2000). Alternatively, studies by Adams *et al.*, (Adams *et al.*), demonstrated differential expression of gene homoeologs in various tissues of *Gossypium hirsutum* individuals at the onset of polyploidisation. This is referred to as subfunctionalisation. and may result in greatly increased pools of

phenotypic and physiological variation upon which selection can act (Adams *et al.*, 2003).

Plants of differing ploidies are often reproductively isolated from one another by strong pre- and post-zygotic reproductive barriers (Ramsey and Schemske, 1998). Consequently, polyploidisation offers an important mechanism by which sympatric speciation (Husband and Sabara, 2004), local adaptation and subsequent range expansion can take place (Levin, 1983). This is aptly illustrated by the eight cytotypes comprising the *F. rubra* complex which range in ploidy from hexaploid to decaploid . The cytotypes occupy differentiated geographic ranges and divergent ecotypes reflecting their widely varying ecological tolerances. For example, ssp. *arctica* inhabits mountainous regions of Scotland and the Shetland Isles whilst ssp. *arenaria* prefers sandy coastal habitats such as those found in Norfolk, Cornwall and Devon (Hubbard, 1984). However, little is known of the progenitor species of each of the *F. rubra* cytotypes and consequently it is also not known whether the ssp. *rubra* and ssp. *commutata* are auto- or allopolyploids.

At the most fundamental level, there are two critical stages in the evolution of species via polyploidisation; the first stage, somewhat obviously, involves polyploid formation, either through the fusion of two unreduced gametes as described above (bilateral polyploidisation) or alternatively through the fusion of an unreduced gamete with a reduced gamete (unilateral polyploidisation) (Harlan and deWet, 1975). The second stage involves the establishment of neopolyploids within populations of their progenitor cytotypes. Whether this initial establishment phase precedes a subsequent range expansion of the species by the novel cytotype or the continued coexistence of the progenitor and neo-cytotypes, it nonetheless involves the overcoming of an establishment barrier.

The very nature of polyploid formation means that novel cytotypes arise within established populations of their progenitor cytotypes. Consequently, at their initial low frequencies neopolyploids may be flooded, in relative terms, with pollen from their progenitor species and yet receive little from individuals of their own cytotype. This generates a frequency dependent fitness barrier referred to as Minority Cytotype Exclusion (MCE) (Levin, 1975). Understanding how neopolyploids overcome this establishment barrier, and how mixed cytotype populations are subsequently maintained, is fundamental to understanding the evolution of polyploids. Indeed, MCE is arguably the most important barrier to polyploid establishment (Levin, 1975). The correspondingly high ploidal levels of the two cytotypes considered here, and the ubiquitous distribution of the octaploid sub-species in comparison to the hexaploid makes it unlikely that either sub-species is a progenitor of the other. Nonetheless, the historically restricted distribution of *F. r. commutata* in the British Isles prior to the extensive seed imports of recent decades (Hubbard, 1984) means that, where it has established itself amongst populations of *F. r. rubra* without specific anthropogenic interference, it is likely to have experienced the same frequency dependent barriers to establishment as any neopolyploid.

Coexistence of cytotypes within populations is a common phenomenon amongst species and studies by Burton & Husband, (Burton and Husband, 1999), Husband & Schemske, (Husband and Schemske, 2000), and Weiss *et al.*, (Weiss *et al.*, 2002), provide good examples of factors facilitating the establishment and maintenance of mixed cytotype populations. Nonetheless, the total number of studies that have investigated the frequency and distribution of cytotypes in mixed-cytotype populations are still few despite their importance to the understanding of polyploid

evolution (Burton and Husband, 1999). Furthermore, all studies to date have focussed on distributions of cytotypes at the medium to large scale.

Examples of exclusively polyploid mixed-cytotype populations are also apparently rare, although some have been described (for example the hexaploid/enneaploid populations of *Andropogon geradii* (Poaceae) described by Keller & Davis, (Keller and Davis, 1999)). Hence the Park Grass populations of our study provide a unique opportunity to investigate the fine-scale distribution of cytotypes across a highly ecologically differentiated environment.

The maintenance of mixed cytotype populations is governed by two principle factors; ecological sorting in heterogeneous environments (Burton and Husband, 1999) and, as with the initial establishment of novel cytotypes, frequency-dependent mating success (Husband and Schemske, 2000; Levin, 1975).

Functional divergence of duplicate genes may allow for differentiation of cytotypes in terms of their ecological tolerances. Thus, in a heterogeneous environment, the potential exists for a novel-cytotype to hold a selective advantage over an established cytotype within some proportion of the broadly suitable habitats. This may allow the minority cytotype to overcome its frequency related disadvantage, and where habitat type has a strong influence on relative fitnesses, monotypic populations of ecologically differentiated cytotypes may exist (Burton and Husband, 1999). The degree to which ecological sorting governs cytotype distributions and the maintenance of mixed cytotype populations is also dependent on other factors. For example, levels of allelic diversity in progenitor cytotypes may influence the generation of beneficial gene combinations in autopolyploids (Jackson, 1982; Soltis and Soltis, 1993). Hence, low levels of allelic diversity may result in limited differentiation in the ecological tolerances exhibited by neo-autopolyploids and their progenitors.

In a similar manner, the strength of frequency-dependent mating success can be affected by several factors. These include pre-zygotic mating barriers (Fowler and Levin, 1984) such as asynchrony of flowering phenology, differing pollen performances (Husband *et al.*, 2002) (which serve to reduce the strength of MCE and increase the frequency of intracytotype mating (Husband and Schemske, 2000)) and the recurrent production or immigration of individuals of the minority cytotype (Husband and Schemske, 2000).

Predominate selfing and clumped distributions of polyploids also promote the establishment and co-existence of cytotypes (McCarthy *et al.*, 1995) since these factors also reduce the impact of MCE through promoting intracytotype mating. Yet whilst *F. rubra* is predominately cross-fertilising (Baumann *et al.*, 2000), variation in the tendencies of the two cytotypes considered here toward clumped distributions may impact on the extent to which they occur in sympatry.

It is a commonly held view that chromosome doubling and polyploidisation events render novel cytotypes reproductively isolated from their progenitor species and other related cytotypes (Husband, 2004; Husband *et al.*, 2002). However, the extent to which this is true has yet to be rigorously examined across a broad range of species and few studies have determined the mechanisms by which such barriers to reproduction are enforced (Husband *et al.*, 2002).

The most commonly described barrier to reproduction between diploid and tetraploid cytotypes of the same species is known as Triploid block. This barrier occurs when triploid offspring of diploid-tetraploid crosses are either inviable or cannot be produced at all, consequently preventing the recurrent production of tetraploids via the Triploid bridge (the chance backcrossing of an unreduced triploid gamete with a reduced diploid gamete).

Barriers to hybridisation between higher ploidies have also been observed (Ramsey and Schemske, 1998), though they also remain poorly described. In reality polyploidisation does not strictly prevent hybridisation between cytotypes. In a survey by Husband (2004), triploids or odd numbered cytotypes were observed in some 45% of species examined (although these were often found to occur at low frequencies), whilst an examination of the literature by Ramsey & Schemske (1998) revealed 31.9% fertility in triploids from a range of studies. Indeed, even considerable differences in DNA content between species do not necessarily indicate divergence will prevent hybridisation (Seal, 1983). For example, the 50% variation in DNA content between *Fetsuca drymeja* and *F. scariosa* does not hamper their production of viable hybrid offspring (Borrill, 1972). Hence, potential for hybridisation between ssp. *rubra* and ssp. *commutata* may also exist.

Furthermore, an investigation by Burton & Husband, (Burton and Husband, 1999), into the population structure of diploid and tetraploid cytotypes of *Galax urceolata* revealed not only the presence of triploid hybrids in all populations, (whether these populations were otherwise of mixed-cytotype or mono-cytotypic), but also that no predominately triploid populations existed. One might expect triploid hybrids to exhibit habitat preferences intermediate to their diploid/tetraploid progenitors, and hence that they would predominate in some proportion of habitats where such intermediate preferences are selectively advantageous. Consequently, Burton and Husband (1999) suggest that differentiation in ecological tolerances alone is not responsible for the observed population cytotype structure and that other factors are likely to have played a role.

With this example in mind, should intercytotype hybrids of the *F. rubra* sub-species be detected amongst populations of the Park Grass meadow, knowledge of their

relative frequencies across the meadow may help in determining the forces most influential in governing cytotype distributions.

Research into the large-scale structure of mixed cytotype populations has only really become feasible in the last two decades since the advent of flow cytometry as a tool for determining DNA content and ploidy of plant and animal nuclei. Flow cytometry provides a rapid method for the large scale screening of populations and has been used in both sex determination of individual plants from heterogametic species (Costich *et al.*, 1991; Dolezel and Gohde, 1995) and in the identification of intercytotype hybrids (Huff and Palazzo, 1998). It also provides an accurate means of estimating nuclear genome size and base composition (Dolezel and Novak, 1991), consequently facilitating the assessment of intracytotype variation in DNA content across populations. Such variations in DNA content may represent selective adaptations of populations to differing environments since genome size is strongly influential in plant evolution and adaptation (Bennett, 1972; Dolezel *et al.*, 1998). Correspondingly, intraspecific variation in genome size has been observed across geographically isolated populations many times (Dolezel and Gohde, 1995), initially through the comparison of total lengths of chromosome sets and then, from the 1960s onwards, via the more direct measurements we are familiar with today (Levin, 2002). However, whether intracytotype variation in DNA content occurs at the fine-scale, such as across the geographically proximate sub-plots of the PGE, remains to be seen. Habitat heterogeneity across the Park Grass meadow may well be strong, but gene flow over such short distances, especially in a wind pollinated species, is also likely to be high and may prevent inter-population differentiation.

Alternatively, genome size may be a plastic trait subject to alteration in response to environmental or developmental stimuli. We do not know whether this is the case in

Festuca rubra, though the results of several studies seem to suggest it to occur in other species (Dolezel *et al.*, 1998). Hence, should variation be detected across populations of the PGE this might reflect some plastic response to environmental or developmental stimuli. On the other hand, it might be taken to suggest local adaptation, either in response to fine-scale habitat heterogeneity or contrasting rates of environmental change.

In Chapter 2 analyses of qualitative species composition data sets representing past and present communities from each of the six populations considered in this study are described. These have provided information on each population's perceived rate of environmental change. To date, rate of environmental change does not appear to have been investigated as a factor contributing to the observed distribution of cytotypes. However, in addition to identifying any monocytotypic populations, the effects of differing rates of environmental change on cytotype frequencies, distributions and intrapopulation variations in DNA content are considered in this Chapter. Furthermore, this study has access to highly detailed information on the relative inputs of organic and inorganic nutrients and soil conditions amongst the environments within which the study populations reside, the effects of which are also considered.

Methods

Special efforts have been made to provide depth to the description of quality control and running procedures employed to ensure accurate determination of each individual's nuclear DNA content. This has been done to facilitate future research into mixed cytotype populations of the Park Grass meadow without unnecessary repetition of the costly efforts undertaken here to obtain information relating to appropriate

methodologies; the vast majority of papers resulting from plant nuclear DNA content analyses fail to provide indications of the number of replicate runs carried out per sample. Information relating to the use of appropriate internal DNA standards is also often lacking, as are descriptions of instrument accuracy checks throughout the course of experimental analyses.

However, since these methodologies are lengthy, they are only covered briefly in this Chapter and described in detail in Appendix 5.

Sample collection: Fresh leaf material (2-3 leaf blades) was taken from thirty plants within each of the six sub-plots of the PGE previously sampled for genotyping analysis (sub-plots 17a, 17d, 13a, 13d, 10b and 16d). Each of the individuals selected for sampling were no less than 2m apart from one another. This served to minimise risk of sampling from any single clone more than once. Only 20-30 samples were collected at any one time. These were stored on ice whilst in transit and then kept in the fridge at 4°C until required for ploidy analysis. Samples were analysed within seven days of collection to minimise degradation of nuclear material.

Staining plant nuclei: Cell nuclei were isolated from each sample and stained using a 'CyStain PI absolute P - plant nuclei isolation and staining kit' (Partec; Germany); a small quantity of leaf material (~0.5cm^2) was chopped using a razor blade in 500µl of extraction buffer provided in the kit. After approximately one minute's incubation the extracted nuclei were filtered through a 50µm 'CellTrics' filter (Partec; Germany) and stained in 2ml of a propidium iodide based staining solution (again provided in the isolation and staining kit) for at least one hour prior to running on a Becton Dickinson FACSCalibur flow cytometer.

More specific directions for sample preparation using the 'plant nuclei isolation and staining kit' referred to above can be found in the manufacturer's protocol.

Estimating rates of intercytotype gene flow: The probability of hybridisation between two cytotypes is dependent of their observed relative frequencies within a population (Husband and Sabara, 2004). Hence, the degree of reproductive isolation between *F. r.* ssp. *rubra* and ssp. *commutata* may be estimated using the assumption that the expected frequency of hybrid offspring, under a scenario of random mating, is two times the product of hexaploid and octaploid frequencies.

Results

DNA content analyses were carried out on a total of 180 individuals; 30 individuals from each of six sub-plots on the Park Grass meadow previously chosen in Chapter 2. Of these 180 individuals, the vast majority (96%) proved to represent the octaploid cytotype, *F. rubra* ssp. *rubra*. The remaining 4% of individuals were either of the hexaploid cytotype, *F. rubra* ssp. *commutata*, or were intermediate in DNA content to the hexaploid and octaploid cytotype DNA ranges reported by Huff and Palazzo (1998).

At the sub-plot level, four of the six populations consisted of a single cytotype which on each occasion was represented by the octaploid ssp. *rubra*. These monocytotypic sub-plots were 17a, 17d, 13d and 16d. Sub-plot 13a, however, was shown to hold a population of mixed cytotype, with five of the 30 individuals screened (17%) representing the hexaploid cytotype. Of the remaining 25 individuals screened from sub-plot 13a, two (7%) had nuclear DNA contents that were intermediate in value to

the DNA content ranges reported by Huff & Palazzo, (1998). The same is true for a single individual, representing an estimated 3% of the sub-plot 10b population which was otherwise monomorphic for the octaploid cytotype.

A comparison of the octaploid nuclear DNA contents gathered from each population in this study (See Appendix 6) revealed significant variation across sub-plots of the Park Grass Experiment ($F_{2,27} = 7.79; p<0.001$) with the populations of sub-plots 16d, 13d and 17d exhibiting comparable average DNA contents that are significantly greater than those of sub-plots 10b, 13a and 17a (see Figure 4.1). Of the three populations exhibiting the lower average DNA contents, the sub-plot 10b value is comparable to that of sub-plot 13a, but still significantly greater than the value for the sub-plot 17a population which holds the lowest value.

Associations were investigated between average DNA contents exhibited by the six populations for the octaploid cytotype and those environmental variables for which data are available. These variables were the rates of application of N, P, K, Na, Mg as well as soil pH (as measured in 1991). Rate of environmental change was also considered as a potential variable with which average population DNA contents for the octaploid cytotype may be correlated.

The results of these investigations revealed there to be no apparent association between the average DNA content for a population and any of the environmental variables considered other than soil pH. This potential correlation between soil pH and nuclear DNA content of octaploid cytotype individuals is illustrated in Figure 4.2 below.

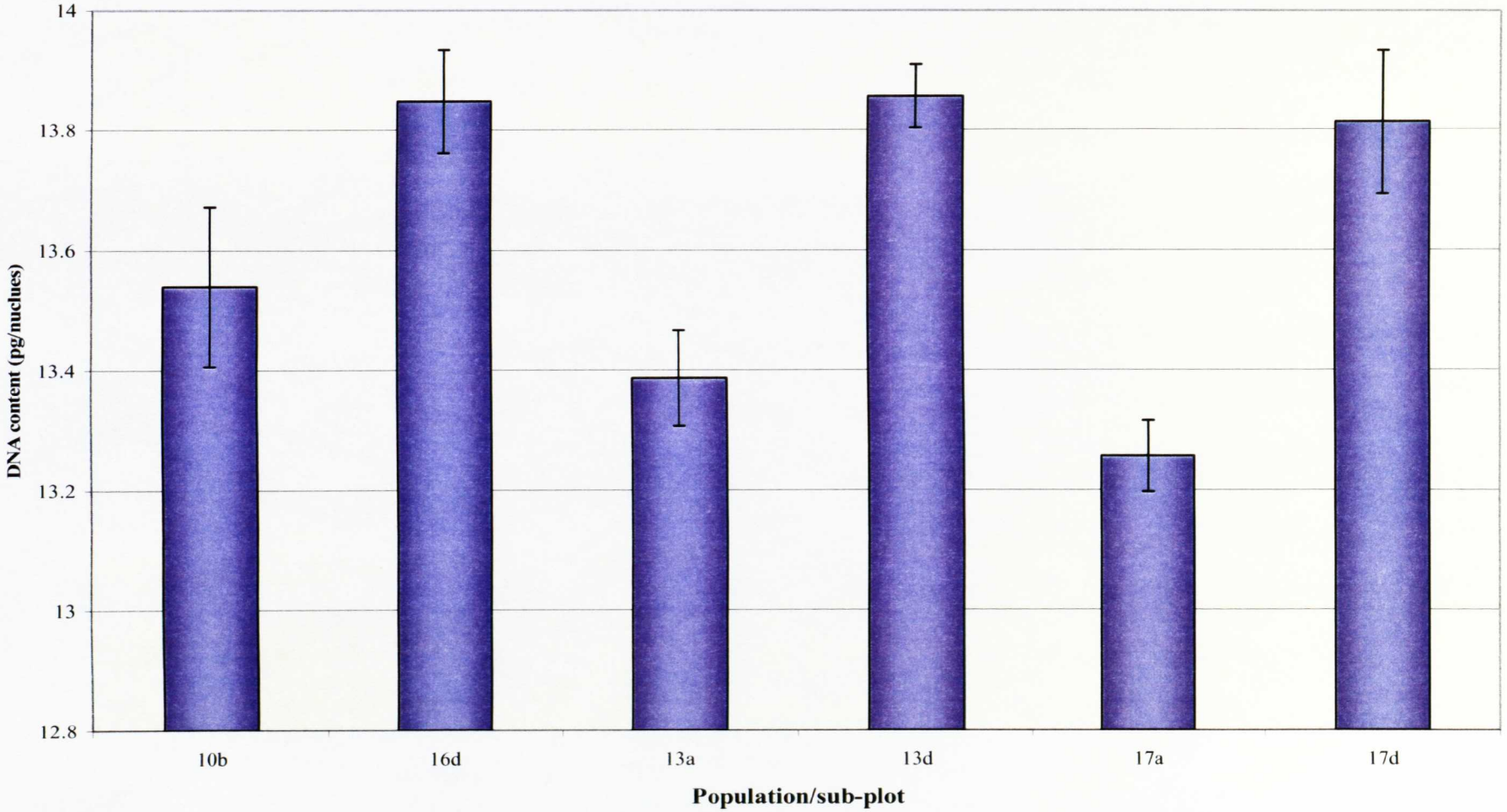


Figure 4.1: Average nuclear DNA content of octaploid cytotype populations on the Park Grass meadow

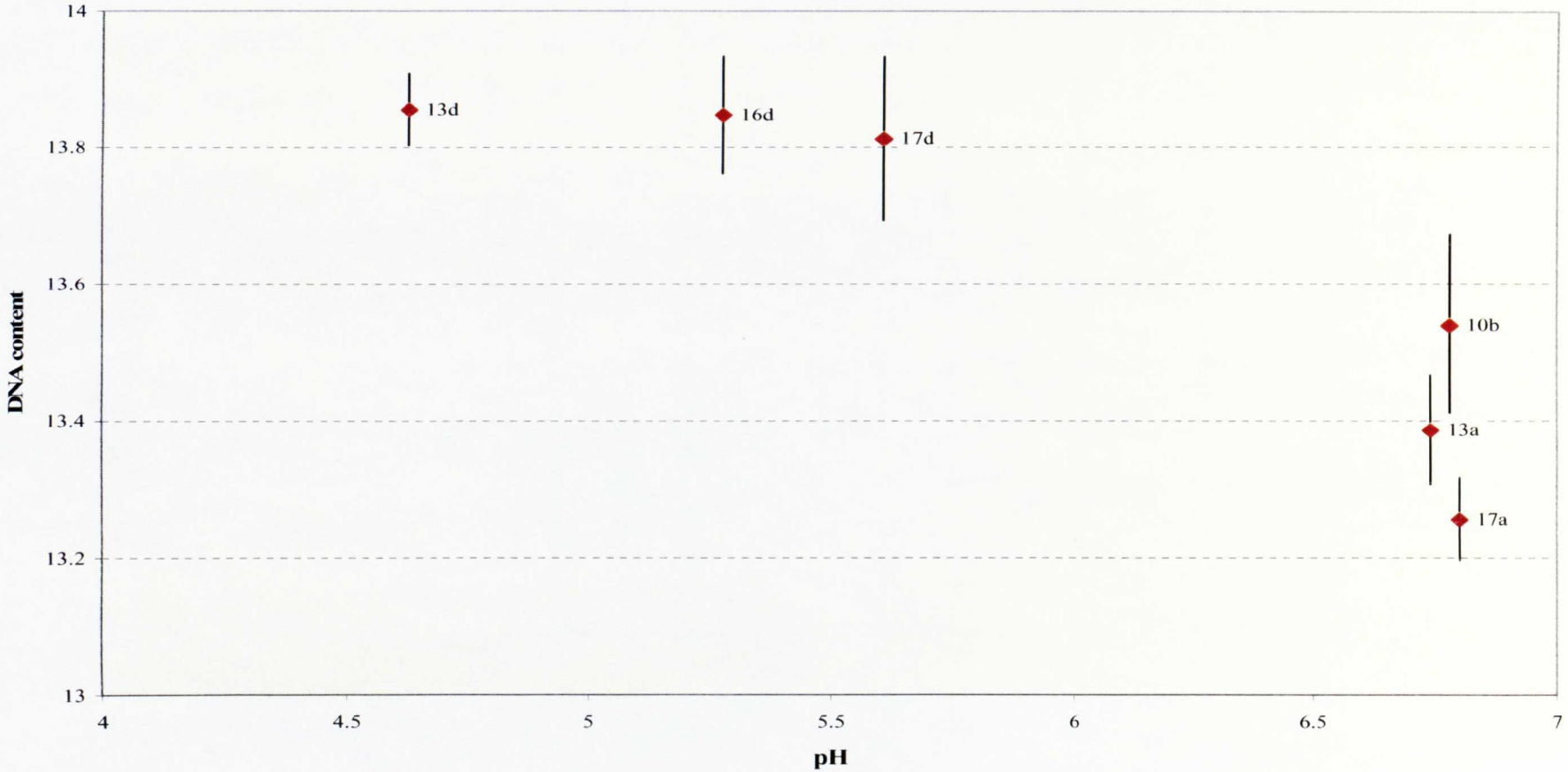


Figure 4.2: Average DNA contents of octaploid cytotype populations on the Park Grass meadow plotted against pH. Data points are plotted with associated standard error bars. pH values for each of the six sub-plots were provided by Rothamsted Research Station and were measured in 1991.

Furthermore, the average DNA content values of the six sub-plot populations, and a further average value calculated to represent the six populations as a whole, were all noticeably less than the mean nuclear DNA value reported for this cytotype by Huff & Palazzo (1998). Unfortunately, ANOVA could not be applied to test if these differences were significant owing to the values from which Huff & Palazzo (1998) calculated their averaged nuclear DNA content for the octaploid cytotype no longer being available (Huff, *pers. comm.*).

Discounting one individual outlier, the *range* in DNA contents for individuals of the octaploid cytotype on Park Grass does, however, appear to be in accordance with that reported by Huff & Palazzo (1998) (see Table 4.1). Nonetheless, the single outlier, an individual from sub-plot 10b with a nuclear DNA content of 15.68pg, falls outside the upper limit of this range. This individual's nuclear DNA content is 0.15pg higher than the largest value reported by Huff & Palazzo (1998).

As already mentioned, the hexaploid cytotype is represented by only five individuals from the sub-plot 13a population, limiting the application of meaningful statistical analyses. Nonetheless, both the mean and range in DNA contents of these five hexaploids are in accordance with the values reported by Huff and Palazzo (1998) for *F. r. commutata* (see Table 4.2).

Table 4.1: Mean and range of DNA contents (pg/nucleus) of *F. r. rubra* individuals from selected sub-plots of the Park Grass Experiment.

Population	mean	low	high
13a	13.39	12.57	13.98
13d	13.86	13.14	14.40
17a	13.26	12.76	14.10
17d	13.81	12.73	15.16
10b	13.54	12.61	15.68
16d	13.85	13.10	15.05
Park Grass (all 6 sub-plots combined)	13.62	12.57	15.68
F. r. rubra (as reported by Huff & Palazzo, 1998)	14.27	12.54	15.53

N.B: Also included are the comparative values reported by Huff & Palazzo (1998) averaged across eight populations from two geographic locations in the United States along with values representing the six Park grass sub-plots as a single population.

Table 4.2: Mean and range of DNA contents (pg/nucleus) of *F. r. commutata* individuals from selected sub-plots of the Park Grass Experiment.

Population	**mean**	**low**	**high**
13a	12.00	11.82	12.31
F. r. commutata (as reported by Huff & Palazzo, 1998)	11.91	11.24	12.37

N.B: Also included are the comparative values reported by Huff & Palazzo (1998) averaged across nine populations from two geographic locations in the United States.

Discussion

Flow cytometric analysis has facilitated the determination of *Festuca rubra* cytotype frequencies amongst populations of six sub-plots on the Park Grass meadow. Four sub-plots have been shown to consist of single cytotype populations represented by the octaploid ssp. *rubra*. One population is a mixture of both the hexaploid and octaploid cytotypes whilst also containing individuals intermediate in DNA content to the ranges reported by Huff and Palazzo (1998) for the two cytotypes, as is also true of the population of sub-plot 10b (though admittedly the individuals of intermediate DNA content are at very low frequencies in the 10b population).

The detection of individuals with DNA contents intermediate to the ranges reported by Huff & Palazzo (1998) for these two cytotypes might suggest introgression of the sub-species. However, such a conclusion should not be drawn without first considering the potential for overlap in cytotype DNA content ranges. With reference once again to the ranges reported by Huff & Palazzo (1998) for the sub-species of this study, the intermediate values which are apparently not exhibited by individuals of either cytotype cover a range of just 0.17pg (from 12.37pg to 12.54pg). However, the DNA content ranges generated by Huff and Palazzo's study, were based on only a small number of populations or 'accessions' representing commercial turfgrass seed-stocks. Consequently, the eight ssp. *rubra* populations they examined are likely to exhibit limited genetic variability when compared to more outbread wild populations. With this is mind it may not appear so extraordinary that the DNA content values from 180 wild individuals, sampled from within a highly heterogeneous environment, should reveal a relatively minor extension to the previously reported range in genome sizes (see Figure 4.3). This extension to the DNA content range of the octaploid

cytotype was observed in a single individual from sub-plot 10b and represents an increase to the upper value by 0.15pg. The more important point here, however, is that should such an extension to the lower limit of the DNA content range for the octaploid cytotype also exist within out sample set, we might mistake this as being indicative of intercytotype hybridisation. This is not to say that intercytotype hybrids do not exist, merely that there is potential for the true lower limits of the octaploid DNA content range to overlap that of any intercytotype hybrids and *vice versa.* Hence, the observed range in octaploid genome sizes restricts us, in a similar way as the results of the previous chapter, from drawing any firm conclusions relating to the occurrence of hybridisation between the two sub-species.

The implications of this finding are that one should not assume gene flow to occur between the two sub-species and, consequently, that the microsatellite dataset relating to the mixed cytotype population of sub-plot 13a cannot be considered to represent a single reproductive unit. On the other hand, it might be argued that the population of sub-plot 10b, which also contains an individual with DNA content intermediate to the ranges reported for the hexaploid and octaploid cytotypes, *can* be treated as representing a single reproductive unit; whether the individual in question is of hybrid origin or represents an octaploid individual with a particularly low DNA content would not affect the population's status as a single reproductive unit.

A further possibility is that the individual of 'intermediate' DNA content from sub-plot 10b is a hexaploid exhibiting a particularly high DNA content. However, this would seem to be the least likely scenario; the individual in question has a DNA content of 12.49pg/nucleus. This is 0.08pg less than the nearest octaploid individual examined from the Park Grass plots, but 0.18pg greater than the nearest hexaploid individual. Further comparison with the ranges reported by Huff & Palazzo (1998)

show the 'intermediate' individual has a DNA content just 0.05pg less than their minimum octaploid value, but 0.12pg greater than their maximum hexaploid value. Of course the possibility that the individual is hexaploid and has a particularly large DNA content cannot be ruled out altogether, but even if this were the case the frequency of hexaploids within the population of sub-plot 10b would be so low that it would be unlikely to have any significant influence on results gained from treating the associated microsatellite dataset as purely octaploid.

The detection of a mixed cytotype population of *Festuca rubra* on sub-plot 13a is of great interest and significance since this finding is contrary to the theoretical predictions of frequency dependent selection (Levin, 1975). The fact that only one of the six sub-plots examined contains hexaploid individuals suggests that ecological sorting within a heterogeneous environment is facilitating the coexistence of cytotypes on the Park Grass meadow rather than some pre-zygotic reproductive isolation mechanism: if the overcoming of frequency dependent selection and subsequent coexistence of cytotypes were facilitated, for instance, by differences in flowering phenology, we might expect mixed cytotype populations to occur on a greater proportion of sub-plots than observed.

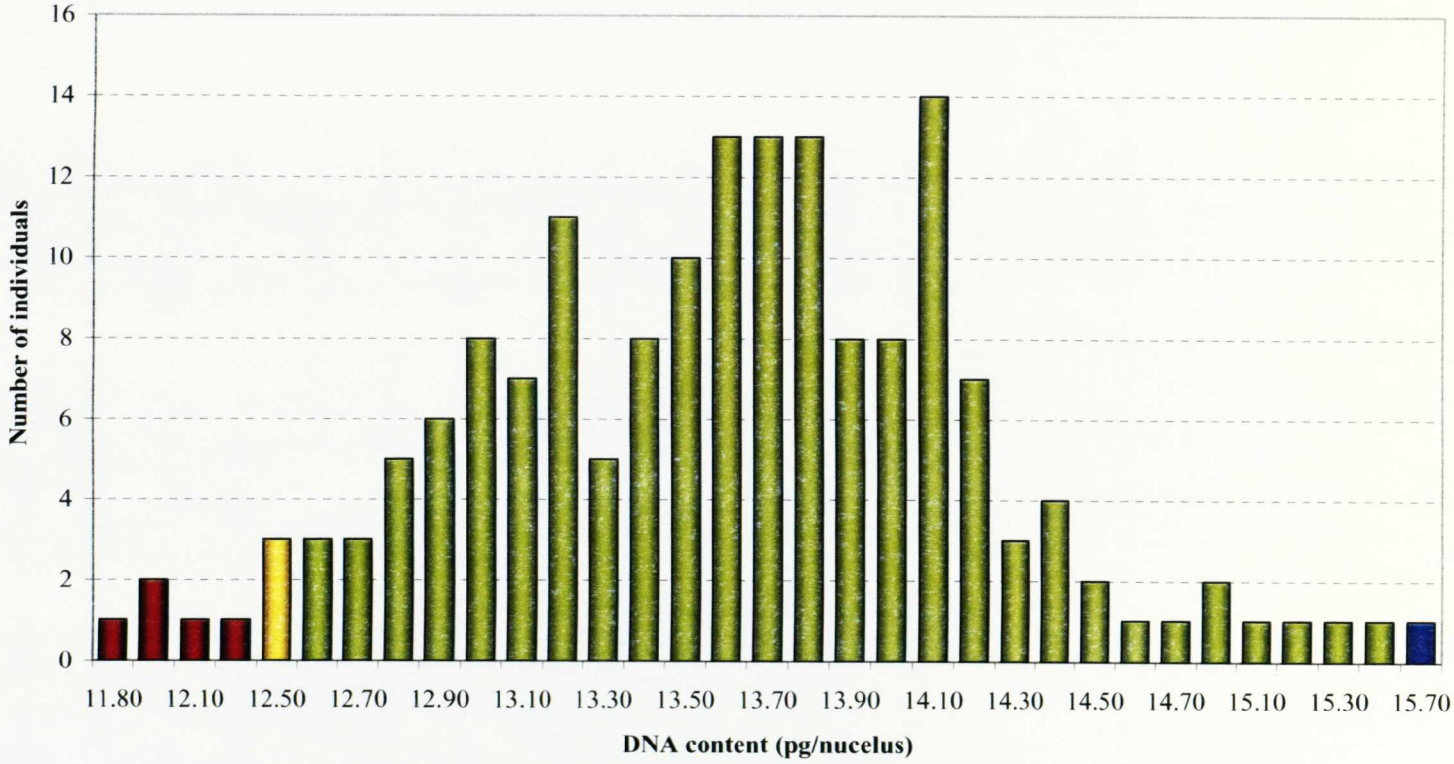


Figure 4.3: Frequency of Festuca rubra nuclear DNA content values across six populations of the Park Grass Experiment. Brown bars represent individuals of hexaploid cytotype. The yellow bar represents individuals of intermediate DNA content to the hexaploid and octaploid values reported by Huff and Palazzo (1998). Green bars represent octaploid individuals within the DNA content range reported by Huff & Palazzo. The blue bar represents the single octaploid individual found with a DNA content value outside that of the octaploid range reported by Huff and Palazzo.

Constituting just 17% of the *F. rubra* composition on sub-plot 13a, the hexaploid ssp. *commutata* is clearly the minority cytotype. Hence, overcoming the frequency dependent mating barrier that may otherwise have led to its exclusion from the population might be facilitated by an ability of hexaploid individuals to exhibit greater fitness than their octaploid counterparts within the subtly distinct community and environment generated on this sub-plot through the application of specific fertilizer treatments. Alternatively, if temporal differentiation were to exist between ssp. *rubra* and ssp. *commutata*, perhaps induced by specific environmental or developmental stimuli, this might also account for their coexistence within selected habitats of the meadow. Indeed, it has been suggested that the rapid rate of leaf growth observed amongst individuals of the octaploid ssp. *rubra* in early spring (Grime, 1985) brings about temporal niche differentiation from species such as *Agrostis capillaris* with which it is otherwise in close competition (Grime *et al.*, 1988).

Sub-plot 13d, which we have shown to hold a single-cytotype population of ssp. *rubra*, has of course received the same fertilizer applications as 13a. However, the withholding of liming treatments to this sub-plot has brought about significant soil acidification and associated changes in community structure and environmental conditions. The communities and environmental conditions within sub-plots 13b and 13c, on the other hand, are likely to be far less different from those of 13a. Consequently, further examination of these populations along with others from more ecologically diverse sub-plots would help in determining if ecological sorting is a genuine factor contributing to cytotype coexistence. If this were shown to be the case, such investigations would also help in determining the breadth of environmental conditions and range of community structures within which the hexaploid cytotype exhibits comparatively elevated fitness.

Of course, ecological sorting does not necessarily occur to the exclusion of factors contributing to reproductive isolation. Hence, further investigations into pollen competition between the two sub-species' and their flowering phenologies would also help in determining the influence, if any, of reproductive isolating mechanisms in the maintenance of the mixed cytotype population.

Statistical analysis of DNA content values revealed there to be significant variation among populations of the octaploid cytotype within the Park Grass meadow. This may seem surprising considering the predominately outbreeding, wind pollinated nature of ssp. *rubra*. It is also noticeable that the populations of subplots 17d, 13d and 16d, which exhibit comparably high average DNA content values relative to the remaining Park Grass populations examined, are all found on unlimed sub-plots. Whilst these unlimed sub-plots have received differing fertilizer treatments, they have all undergone significant soil acidification as a result of the withholding of liming applications. Figure 4.2, shown in the previous section, illustrates a potential correlation between DNA content of octaploid cytotype individuals and soil pH. The observed correlation is obviously based on just a small sample size of six populations. Nonetheless, it may represent a common adaptive mechanism exhibited by individuals of this sub-species to the decreased soil acidification, perhaps in the form of a plastic developmental response, or as a result of local adaptation in response to common selective pressures.

It is also interesting that such population differentiation can occur over such small distances. The population of sub-plot 17a is significantly differentiated from that of 17d in terms of individual DNA contents and the same is true for the populations of sub-plots 13a and 13d. Yet the distance between the population pairs is just 20m suggesting population differentiation to have occurred as a result of strong habitat

heterogeneity and differing selective pressures rather than random genetic drift. What is more, the observed differentiation is likely to have occurred within a period of just 101 years since the division of plots 17 and 13 into sub-plots and the commencement of liming.

A noticeable difference was also observed between the average nuclear DNA content value calculated from octaploid individuals across the Park Grass meadow as a whole, and that reported by Huff and Palazzo (1998) from their examination of seed stock accessions in the United States (Ave. nuclear DNA contents for Park Grass individuals and US seed stock samples are 13.62 and 14.27pg/nucleus respectively). As explained in the previous section, statistical tests could not be applied to determine if this difference is significant due to the values from which Huff & Palazzo (1998) calculated their averaged nuclear DNA content value no longer being available. Nonetheless, significant or otherwise, considering the geographic separation of the two sample sets, the observed difference in average nuclear DNA contents is not wholly unexpected and may be the result of chance genetic events between the geographically isolated populations or adaptive differences between the populations.

In conclusion, flow cytometric analysis of *Festuca rubra* on sub-plots of the Park Grass meadow has enabled identification of populations of mixed cytotype and of single cytotype. Consequently, it has also been possible to speculate on the mechanisms facilitating the existence and maintenance of the mixed cytotype population discovered on sub-plot 13a. Further investigations into temporal stability of the cytotype ratio of this population over coming years will allow for additional inferences to be made relating to whether the mixed cytotype population has reached an equilibrium state, or whether the observations of this study are the result of chance

stochastic events from which the population is returning to, or moving toward, a monocytotypic state as seen in the five other populations examined.

The detection of significant variation in DNA contents among populations of the Park Grass meadow has provided another example of fine scale population differentiation. This is likely to be a consequence of habitat heterogeneity and the differing selective pressures imposed on populations. As with the studies on *Anthoxanthum odoratum*, (Snaydon and Davies, 1976), the investigations described here have shown that, at least in species with high levels of standing genetic diversity such as *Festuca rubra*, population differentiation can occur not only over short distances, but also over short periods of time.

Of the 30 individuals screened from each of the six populations of this study, ssp. *commutata* was not detected in five. However, this finding does not exclude the possibility that ssp. *commutata* may be present at extremely low frequencies in these five populations. Nonetheless, at such low frequencies the influence of ssp. *commutata* on investigations into the relative ratios of clonal to sexual reproduction amongst populations of ssp. *rubra*, which is the subject of the next Chapter, is likely to be negligible.

Chapter 5

Relative rates of clonal versus sexual recruitment in *Festuca rubra* ssp. *rubra*.

Introduction

Various studies have investigated the effects of spatial environmental heterogeneity on clonal/sexual reproductive allocation in plant species (Mandujano *et al.*, 1998; Skalova *et al.*, 1997; Wijesinghe and Hutchings, 1997), yet few have considered the influence of temporal heterogeneity (though see Xie, (Xie *et al.*)). This may not seem surprising considering the time and resources required for determining rates of environmental change within different habitats over ecologically meaningful time-frames. Nonetheless, having had access to data held on the Electronic Rothamsted Archive, we have identified sub-plots of the PGE that have experienced greatly contrasting rates of environmental change. Grasslands in general are known to exhibit high levels of spatiotemporal variability (Silvertown *et al.*, 1988). For purposes of comparison, communities have been identified in this study that have experienced higher rates of environmental variation whilst others, that have been shown to have witnessed relatively stable conditions, have also been chosen for investigation. Consequently, this study into *F. rubra* populations on Park Grass represents a rare opportunity to investigate the hypothesis that environmental variation favours the evolutionary maintenance of sexual reproduction and recombination.

Those sub-plots of the PGE that have been shown to have experienced limited environmental change effectively represent stable environments. Within such environments it might be expected that selection will favour clonal reproduction of genotypes displaying high levels of fitness; under stable environmental conditions clonal reproduction ensures individuals of each generation will exhibit the same high levels of fitness as previous generations. Sexual reproduction, on the other hand, often breaks down favourable gene combinations through recombination (Lewis, 1987)

such that the local adaptations of individuals to their specific environments may be lost in subsequent generations at a faster rate than new genotypes of high-fitness are generated.

Clonal propagation is strongly influenced by spatial and temporal heterogeneity (Wijesinghe and Hutchings, 1997), and in rapidly changing and unpredictable environments one would expect sexual reproduction to have an advantage over clonal reproduction in terms of genetic variation and rate of evolution. In response to environmental change, sex can produce genotypes of high fitness (through recombination) at a faster rate than clonal reproduction (which is dependent on mutation events for the generation of novel genotypes). Consequently, in rapidly changing environments, selection may favour sexual reproduction.

Nonetheless, such expectations may be confounded if, for example, selection in an unpredictable environment were particularly strong. This might result in the genetic variation produced through sexual reproduction and recombination proving insufficient to cover the range of genotypes required. Similarly, strong periodic selection may purge a population of genetic variation (Cohan, 1994; Maynard Smith, 1991) such that the remaining variability is inadequate for sexual reproduction to have any advantage.

Variation in reproductive allocation may also be a consequence of the differing plastic responses of genotypes to their environments (van Kleunen *et al.*, 2002). Selection may favour plastic generalist genotypes in temporally heterogeneous environments (Scheiner, 1993), and since recombination will disrupt the genotypes displaying favourable levels of plasticity, selection may instead favour clonal reproduction which ensures preservation of these beneficial traits in the following generation.

Festuca rubra is an ecologically flexible species owing, in part, to high levels of phenotypic plasticity (Grime, 1998) as discussed above. This has been illustrated by the ability of individual clones to exhibit differing strategies of rhizome growth depending on the strength of interspecific competition encountered (Skalova *et al.*, 1997). Ecological flexibility in this species also results from high levels of genetic variation (Grime *et al.*, 1988) which facilitates a quicker response to change (Hamrick and Godt, 1996), as has also been demonstrated with the detection of lead-tolerant genotypes on motorway verges (Atkins *et al.*, 1982). Consequently, the potential exists for selection to favour the relative allocation to sexual versus clonal reproduction in *F. rubra* populations in rapidly changing environments.

The findings described in previous chapters have revealed that, considered as a whole, the *Festuca rubra* population of the Park Grass meadow is represented by two sub-species. However, it has also been determined that, at the sub-plot level, populations within the Park Grass meadow are not necessarily mixed cytotype; the *F. rubra* populations from five of the six sub-plots targeted in this study are monocytotypic for ssp. *rubra*. Only the population of sub-plot 13a has been identified as mixed cytotype. Hence investigations into this population must take into consideration the likelihood that the sample set is represented by individuals of both ssp. *rubra* and ssp. *commutata*.

A combination of nuclear and chloroplast microsatellite markers have been used to distinguish *F. rubra* genotypes and to determine the ratio of genotypes to samples within each of the six populations of this study. This is used as an estimate of the relative frequencies of sexual and clonal reproduction in each population. Many earlier studies concerned with the identification of genets have relied on dominant markers (Tani *et al.*, 1998). However, our extensive preliminary investigations

revealed dominant Inter Simple Sequence Repeat (ISSR) markers to be inappropriate for genotype differentiation in *Festuca rubra* owing to a lack of reproducibility in results from this species. Similarly, comparative studies have shown levels of polymorphism exhibited by dominant allozyme markers to be insufficient for confident distinction of genets within populations of some species (Esselman *et al.*, 1999).

Microsatellite markers, on the other hand, are codominant, often highly polymorphic and have been successfully used in population studies of clonal structure in several species (Ainsworth *et al.*, 2003; Nagamitsu *et al.*, 2004; Schilder *et al.*, 1999). However, early studies revealed low mutation rates at the majority of chloroplast loci and, as a consequence, detecting sufficient amounts of variation required for population-level studies within this particular genome has proved problematic (Provan *et al.*, 2001). Nonetheless, more recent publications have suggested chloroplast marker variation at the intraspecific level to be far more common than originally thought (Harris and Ingram, 1991; Soltis *et al.*, 1992), whilst several studies also describe the detection of polymorphic mononucleotide repeats within the chloroplast genome (Powell and al, 1995; Provan *et al.*, 1996).

Relatively few studies have used microsatellites markers to investigate polyploid species (Saltonstall, 2003), likely due to an inability to determine allele dosage in such individuals, prohibiting the calculation of allele frequencies and expected heterozygosities. The identification of polymorphic chloroplast microsatellite loci offers a means of circumventing this problem, due to the haploid nature of the chloroplast genome, though here such considerations are of secondary interest. The primary focus of this study concerns the discrimination between different genotypes and the estimation of relative ratios of clonally to sexually reproduced individuals

within each population. Yet, in the absence of polymorphic chloroplast loci, it is still possible to calculate the total number of 'allelic phenotypes' (the banding patterns observed at particular loci) (Becher *et al.*, 2000), observed heterozygosities (Saltonstall, 2003) and genetic distances (Tomiuk and Loeschcke, 1996) between populations of high ploidal species. Consequently, attention is given to these basic population statistics at later points in this chapter since information relating to the amount of genetic diversity present within and between sub-plot populations, in the context of external populations, may be of significance to the observed modes of reproduction. Whilst there may be advantages associated with sexual reproduction in rapidly changing environments, if levels of genetic variation amongst local populations are limited, sexual reproduction may not generate the range of genotypes necessary to accommodate environmental variation. Also, if diversity is observed to be particularly low within a population shown to have experienced rapid environmental change, then this might be accounted for by the purging of genetic variation associated with strong, unidirectional selection.

With our investigations focussed on six sub-plots whose populations have been shown to have experienced contrasting rates of environmental change, we aim to determine whether a correlation exists between rate of environmental change and mode of reproduction.

The null hypothesis to be tested is as follows:

The proportions of sexually / clonally recruited individuals do not vary between sub-plot populations of the Park Grass meadow.

Any significant deviation within a population from the null hypothesis may consequently represent a selection bias toward either sexual or clonal recruitment.

In addition to the six sub-plot populations of the PGE, samples were also collected from four geographically isolated meadows across Southern England. This was done because levels of intraspecific polymorphism in *F. rubra* at the targeted loci have not previously been measured. Consequently, should any loci prove monomorphic amongst individuals sampled from the PGE, comparison with other geographically distant populations will allow us to determine whether the observed monomorphism is the result of low variability amongst individuals of the PGE or due to limited polymorphism within the species as a whole at the locus concerned.

A further two small sets of individuals, one representing ssp. *rubra* and one ssp. *commutata*, were also included for genotyping at the microsatellite loci. These samples, which had been previously collected from random plots across the Park Grass meadow as part of the Internal Transcribed Spacer sequence investigations described in Chapter 3, were incorporated into the microsatellite investigations. This was done to provide a preliminary indication as to whether either sub-species possess fixed private alleles at any of the targeted loci that might prove useful for identification purposes.

Methods

Sample collection for microsatellite analysis: Samples were all collected during the spring of 2003. Twenty five samples were collected from each of the six sub-plots of the PGE (150 samples in total) identified in Chapter 2 as being appropriate for investigation in this study. These were sub-plots 16d, 10b, 13a, 13d, 17a and 17d. Sub-plots 10d and 3d, which were included in the statistical analyses of Chapter 2 for

reference purposes, were not sampled owing to the extremely low abundance of *F. rubra*.

Samples were taken every 10cm along 2.5m transects set in the middle of the sub-plots, running parallel to the north-east/south-west boundaries (see Figure 5.1). If an individual could not be found growing directly on a transect line at a particular sampling point, then the nearest individual found growing either side, perpendicular to the transect, was sampled. The distance from the transect line at which individuals were sampled was recorded along with whether they lay to the east or west of the transect line. Few of the sampled individuals lay more than 30cm from their respective transects whilst only a very small number of individuals (<10) had to be collected at distances greater than 50cm.

A further 80 samples were collected from four sites (20 samples per site) across southern England. These were Alpine meadow in Ashridge Park, Hertfordshire (Ash (Grid ref: SP963150)), Frogmore meadow in the Chess valley, Hertfordhsire (Frg (Grid ref: TQ018990)), Cricklade meadow in Wiltshire (Crc (Grid ref: SU094945)), and Pilch Field meadow just outside Milton Keynes, Buckinghamshire (Plch (Grid ref: SP748321). The abbreviations of sample-site names, given in parentheses, will be used in the figures and tables to follow. Sampling was carried out randomly at each site, although to ensure a good representation of the genetic diversity present within each population, individuals were sampled at least 20m apart from one another.

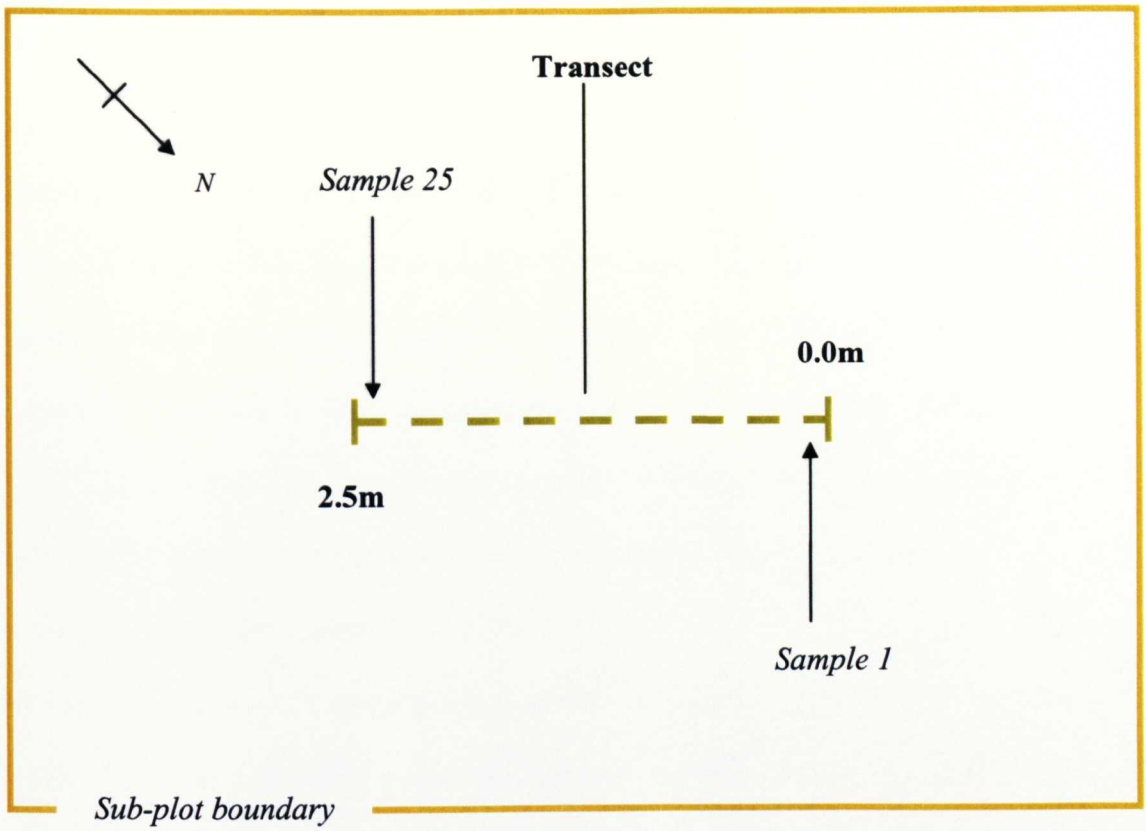


***Figure 5.1*:** Diagram illustrating the positioning of transects through the centre of each sub-plot investigated. Transects were positioned equidistant from the southwest and northeast boundaries of the sub-plots

Due to sampling regulations, rooted plants could not be collected from the Park Grass meadow, and only two or three leaves were taken from each individual sampled. These were stored on ice for transportation back to the lab and subsequent DNA extraction.

DNA extraction: DNA was extracted from fresh leaf material using the modified CTAB protocol described in Chapter 3. Each batch of extractions was carried out with

the inclusion of a negative control and all extraction aliquots were then standardised for concentration to10ng/μl, again as described in Chapter 3.

Identification of suitable primer pairs: The sequences of 50 nuclear microsatellite primer pairs, described by Jones *et al.*, (Jones *et al.*), and originally developed for *Lolium perenne* were kindly supplied by the Plant Biotechnology Centre in Victoria, Australia. 15 of these were screened both for consistency in amplification and generation of expected fragment length products across a sub-set of *F. rubra* samples representing populations from both the Park Grass meadow as well as the four populations sampled external to the PGE .

Primer pairs shown to amplify consistently across a range of individuals from different geographic locations were subsequently screened for polymorphism. Those found to exhibit variability were used in amplification from all sampled individuals, both from the PGE and the four external populations.

A total of five chloroplast primer pairs, originally designed for *Oryza sativa*, (Ishii *et al.*, 2001), were screened in the same way as described above and, once again, those that proved variable were used in amplification from the entire sample set.

Amplification and genotyping of microsatellite loci: Preliminary investigations identified four suitable loci, three nuclear and one chloroplast, at which all samples were amplified.

Amplification via PCR was performed in 10μl volumes using 10ng of extracted nucleic acids, 1.0μl PCR Reaction Buffer (ABgene), 1.5mM $MgCl_2$, 0.4mM dNTP mix (ABgene), 0.5pM of each primer (forward primers were labelled with either FAM or HEX fluorescent dyes), and 0.25U *Taq* polymerase (ABgene). A hot-start PCR

cycle was performed using a Techne Touchgene Gradient PCR machine with the following parameters: an initial denaturation was carried out at 94°C for two minutes followed by thirty five cycles of denaturation at 94°C for one minute, annealling at 64°C for thirty seconds and extension at 72°C for 30 seconds. A further final extension step was performed at 72°C for ten minutes.

Cycling conditions were slightly altered for optimisation of amplification at one of the nuclear loci (LPSSRK10F08) with the annealing temperature reduced to 57°C. Similarly, amplification of the chloroplast locus RCt4 was optimised under the same cycling parameters and cocktails as given above except for the primer concentration which was reduced to 0.25pM of each primer per reaction.

Amplified products were run with a 350bp ROX-labelled internal standard on an ABI 377 automated sequencing facility (Applied Biosystems), using a three second injection rate, at the NERC Molecular Genetics Facility, University of Sheffield.

Data analysis: The octaploid nature of *Festuca rubra* ssp. *rubra* means that anywhere between one and eight bands might be detected at each of the nuclear loci. Estimating relative band strengths and consequently resolving allelic dosage in this situation is likely to be subjective and inaccurate at best, impossible at worst. Consequently, since we are unable to determine the number of copies of each allele within heterozygous individuals at the nuclear loci, it is impossible to determine an individuals' genotype. This is restrictive to our statistical analysis of the microsatellite data. However, it is still possible to calculate the total number of 'allelic-phenotypes', (the array of alleles possessed by an individual at all targeted loci), as are the observed heterozygosities (H_O) for each locus, this being the proportion of individuals carrying heterozygotic allele-phenotypes within populations.

The program F-Dash (Obbard *et al.*, 2003) was used to calculate several statistics from the allele-phenotype data including 'average Shannon-Weaver phenotype diversity within populations', (H '), via the equation below which is averaged (weighted by sample-size) across populations;

$$H' = -\sum_{i=1}^{m} Pi \log(Pi)$$

where m is the number of alleles and p_i is the frequency of the ith phenotype.

The nuclear and chloroplast allele-phenotype data were also analysed using POPDIST (Guldbrandtsen *et al.*, 2000) for the calculation of population genetic distances via the maximum likelihood measure of Tomiuk & Loeschcke, (Tomiuk and Loeschcke, 1996).

The ratio of genotypes to samples within each population was determined and heterogeneity in these relative frequencies across all six populations was tested for using a 'G-Test of replicated goodness of fit' (Sokal and Rohlf, 1995).

Results

The screening of 20 primer pairs on a preliminary sample set revealed three nuclear loci and one chloroplast locus with which it was possible to achieve consistent amplification across the range of genotypes and at which polymorphism was also displayed. The three nuclear loci were LPSSRK08F05 (F05), LPSSRK10F08 (F08) and LPSSRK03B03 (B03) (Jones *et al.*, 2001), whilst the chloroplast locus was RCt4 (Ishii *et al.*, 2001).

The number of alleles observed within our total sample set at each of the nuclear loci was high (see Table 5.1) ranging from 13 to 37. However, the observed number of alleles at the chloroplast locus was just six, although this lower level of variability relative to the nuclear loci might have been predicted due to the haploid nature of the chloroplast genome compared to the hexaploid/octaploid nuclear genomes of the two sub-species.

The high levels of polymorphism at the nuclear microsatellite loci afford a powerful means of distinguishing even closely related genotypes; from amongst a sample set of 245 individuals (N.B. one individual from sub-plot 13a could not be amplified at any of the four loci) between 18 and 161 allelic-phenotypes were exhibited depending on the nuclear locus considered. Observed heterozygosity (H_O) at each of the three nuclear loci was also extremely high and again is likely to be a reflection of the high ploidal levels of the two sub-species.

Table 5.1: Primer information and allelic diversity measures of polymorphic microsatellite loci

LOCUS	PRIMER SEQ (FORWARD / REVERSE)	MAX NO. OF ALLELES / INDIVIDUAL	REPEAT MOTIF	FLOURESCENT LABEL	OBSERVED ALLELE SIZE RANGE	EXPECTED ALLELE SIZE* (BP)	NO. OF ALLELES OBSERVED	NO. OF ALLELIC PHENOTPYES	H_O
LPSSRK08-F05	TGTGCAGTAACT TATGGAACTCTG	8	GA	HEX	283-355	334	37	161	0.98
	TTTGTCTTAACAG CTAGTGGAGCA								
RCt4	ACGGAATTGGAA CTTCTTTGG	1	A	FAM	79-136	120	6	6	0.0
	AAAAGGAGCCTT GGAATGGT								
LPSSRK10-F08	ACCCTGCCATAC ATAGCATGGTGC	8	CAA	FAM	90-147	135	13	18	0.91
	CTGTTGTGGCTG AGGCTGGAAGAA								
LPSSRK03-B03	GGGAATCTGGCA GAAGTATCACGT	8	CA	FAM	242-307	296	35	107	0.86
	GAAGATCTGGCC AAGTCTAATCCG								

N.B: Allelic statistics are based the total sample set of *Festuca rubra* individuals, including those of the mixed cytotype population of sub-plot 13a (n = 24). Observed heterozygosity values (H_O) represent the proportion of individuals positively amplified at each locus and displaying heterozygotic allele phenotypes. * = as reported by the Plant Biotechnology Centre in Victoria, Australia.

As already explained, due to the high ploidies of the two sub-species considered here, we are unable to determine allelic dosage at any of the nuclear loci. Hence, it is not possible to determine an individual's genotype. Nonetheless, we are still able to *distinguish* between genotypes through the determination of each individual's allelic-phenotype across all four loci.

Table 5.2 displays the number of allelic-phenotypes identified within the sample sets of each population. The four populations external to the PGE display ratios of samples to genotypes at, or close to, 1:1. This might be expected owing to the physical separation of samples (≥20m) collected from each of these populations. Similarly, the eight *F. r. rubra* and eight *F. r. commutata* individuals sampled from various points across the Park Grass meadow also display 1:1 ratios of samples to genotypes.

However, our interest lies in the relative proportions of clonally to sexually reproduced individuals within the Park Grass populations. Here, these proportions are unequal within the sample sets of each population investigated. This may represent differing rates of clonal and sexual recruitment.

Along the transects of sub-plots 17a and 13d there are far fewer genotypes than samples and correspondingly greater proportions of clonally than sexually recruited individuals. In contrast, within the populations of the remaining four sub-plots investigated, the number of sexually reproduced individuals outweighs the number of individuals of clonal origin.

***Table 5.2*:** Statistics relating to the number of clonal individuals and genotypes detected within each population

POPULATION	NO. OF SAMPLES	NO. OF GENOTYPES	NO. OF CLONAL INDIVIDUALS	RATIO OF GENOTYPES TO SAMPLES
10b	25	20	9	0.8
16d	25	20	9	0.8
17a	25	15	16	0.6
17d	25	24	2	0.96
13a	24	20	7	0.83
13d	25	14	17	0.56
Plch	20	20	0	1.0
Crc	20	20	0	1.0
Frg	20	19	2	0.95
Ash	20	19	2	0.95
Frc	8	8	0	1.0
Frr	8	8	0	1.0

N.B: *Frc* and *Frr* refer to two small sample sets taken from across the PGE to represent the *F. rubra* sub-species of this study in isolation (*Frc* = *F. r. commutata*, *Frr* = *F. r. rubra*). Clonal individuals were identified through their sharing of allelic phenotypes with other individuals.

A *G*-test was carried out to determine whether there is significant heterogeneity in the number of clonal and sexual individuals across the six populations (see Table 5.3). This revealed that the observed frequencies were indeed significantly heterogeneous; ($G = 28.61$, $P < 2.76 \times 10^{-5}$, d.f. = 5).

The populations of sub-plots 17a and 13d have the highest proportions of clonally reproduced individuals, suggesting a selection bias favouring clonal recruitment. Conversely, the opposite is true of the remaining four populations within each of which there may be a selection bias favouring sexual recruitment.

The graph displayed in Figure 5.2, below, illustrates a potential correlation between rate of environmental change (as determined in Chapter 2) on those five sub-plots with monospecific populations of ssp. *rubra*, and the mode of reproduction favoured by selection. Unfortunately, the low population sample size precludes application of meaningful regression analyses that would otherwise inform us whether the observed potential correlation is significant.

Table 5.3: Results of the *G*-Test of replicated goodness of fit applied to the observed proportions of sexual and clonal individuals determined for each population.

Sub-plot	**No. of sexual individuals**	**No. of clonal individuals**	**No. of samples**
10b	16	9	25
16d	16	9	25
17a	9	16	25
17d	23	2	25
13a	17	7	24
13d	8	17	25
	d.f. = **5**	***G* = 28.61**	
	P = **2.76×10^{-5}**		

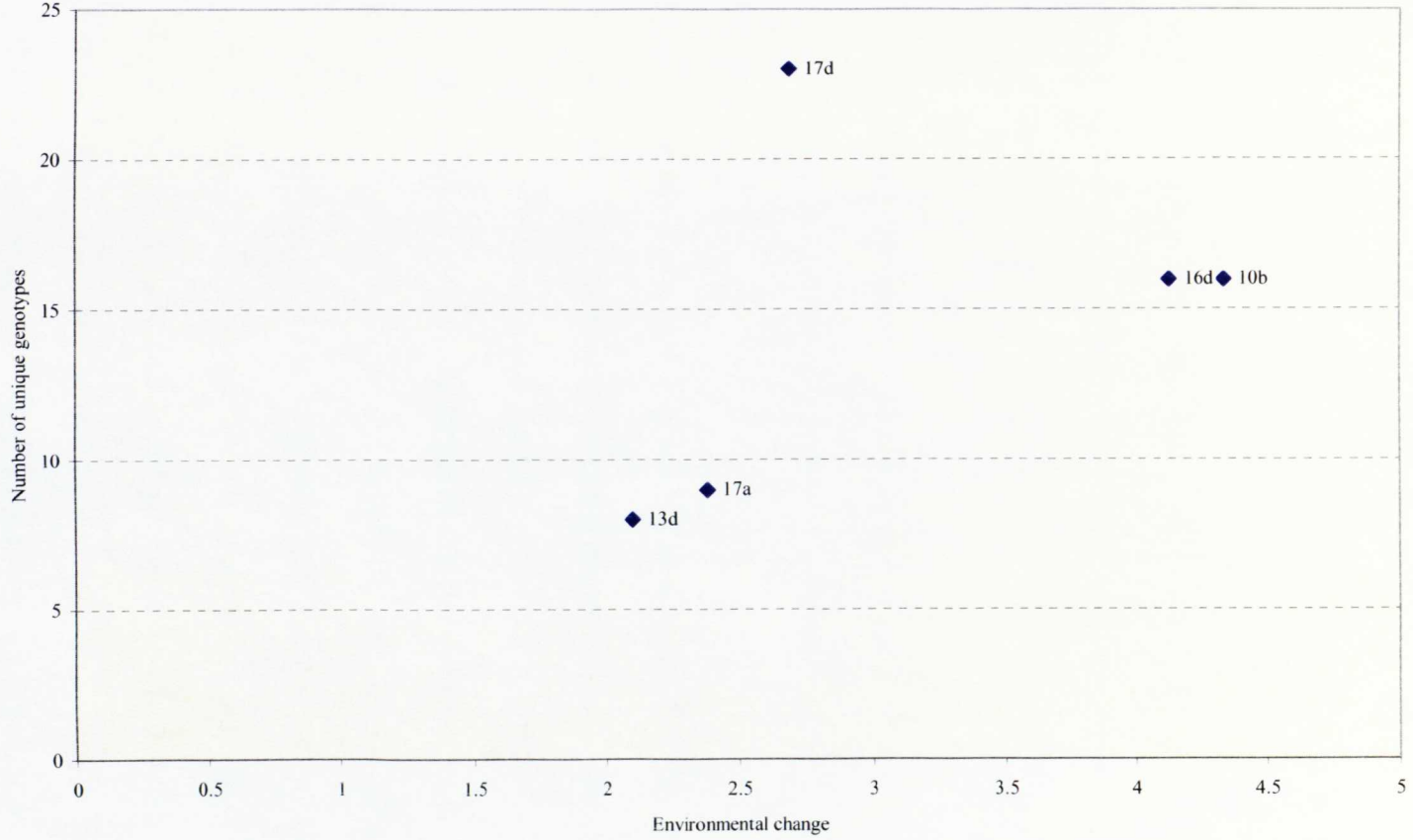


Figure 5.2: Environmental change over the past ~25 years against the number of sexual individuals identified within each population. Only those five populations that are monospecific for *F. r.* ssp. *rubra* are included here. The values for environmental change, as estimated via the Mean Character Difference metric, on each sub-plot refer to the period from the mid 1970's to 2000. 'Sexual individuals' were identified through their exhibiting of unique allelic phenotypes.

Table 5.4 lists the average Shannon-Weaver phenotype diversity measures calculated within populations both at individual loci and averaged across all loci. As might be expected, considering the total number of allelic-phenotypes exhibited by each locus, F05 possesses both the highest measure of phenotypic diversity averaged across the populations, and the highest phenotypic diversity value of all the loci for any single population (*H'* for locus F05 is 3.00 within the Pilch Field population). Consequently, the allelic phenotype data from locus F05 is seen to have the greatest effect on measures of phenotype diversity both within and across populations and has the greatest diagnostic strength of all four of the loci in terms of distinguishing the *F. rubra* genotypes.

In contrast, chloroplast locus RCt4 has by far the least effect of all four loci on the phenotype diversity measures and the weakest influence in the distinction of genotypes.

The remaining two loci are slightly more variable than locus F05 in their effects on overall estimates of phenotype diversity (estimates for locus F08 range from 0.46 to 1.75 depending on the population considered), though both are still informative.

Averaged across all four loci, the Shannon-Weaver estimates (which are weighted for sample size), displayed in Figure 5.3 below, suggest that the Pilch Field, Frogmore and Ashridge populations are most diverse. This is not wholly unexpected, since the random sampling of individuals from those populations external to the PGE is likely to provide a better representation of the genetic diversity present within a population than the sampling of individuals along a 2.5m transect.

Nonetheless, within-population diversity estimates are still higher in the populations of sub-plots 10b and 17d than in the Cricklade meadow population, though admittedly the estimate for the sub-plot 13d population is comparatively low.

The low estimates of within-population diversity from the *Frc* and *Frr* 'populations' are likely to be a consequence of the small sample number.

Table 5.4: Shannon-Weaver phenotype diversity within populations calculated for each locus and as an average across all loci.

	Shannon-Weaver phenotype diversity					
Population	**Sample number**	**F05**	**F08**	**B03**	**RCt4**	**Ave. across all loci**
10b	25	2.14	1.39	2.24	0.36	1.53
16d	25	2.59	1.01	1.85	0.00	1.37
13a	24	2.60	1.75	1.79	0.18	1.56
13d	25	2.25	0.46	1.81	0.00	1.10
17a	25	2.08	1.51	2.28	0.00	1.47
17d	25	2.67	1.20	2.67	0.35	1.71
Plch	20	3.00	1.33	2.86	0.43	1.95
Crc	20	2.77	1.03	2.48	0.00	1.58
Frg	20	2.89	1.71	2.72	0.22	1.90
Ash	20	2.52	1.31	2.72	0.66	1.79
Frr	8	1.95	0.96	1.75	0.00	1.16
Frc	8	2.08	0.80	2.08	0.00	1.25
Average		**2.50**	**1.24**	**2.32**	**0.19**	**1.56**

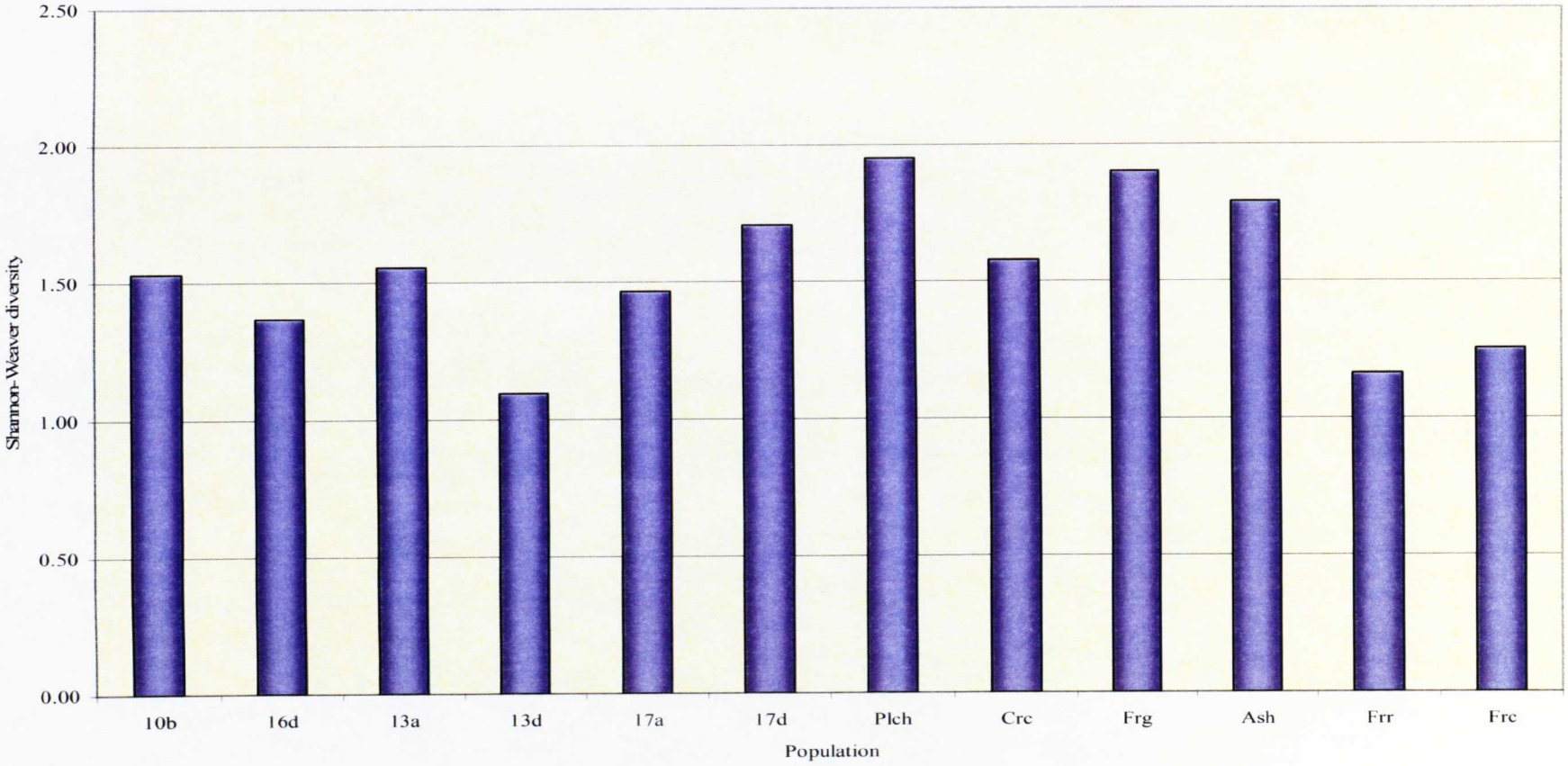


***Figure 5.3*:** Average Shannon-Weaver phenotype diversity within populations across all loci.

The matrix of pairwise genetic distances (as shown in Table 5.5 and graphically depicted in the form of a dendogram in Figure 5.4) reveals some of the highest values to be held by population pairs from the Park Grass meadow. For example, the pairwise genetic distance estimated for the populations of sub-plots 10b and 13a is 0.139 (±0.43). This value is greater than all bar one of those displayed in the entire distance matrix and illustrates the large amount of divergence that has taken place between populations of the Park Grass meadow as a whole.

Three of the four highest genetic distances in the matrix each involve the population of sub-plot 13a in their pairwise comparisons. It might be suggested that this is a result of the mixed cytotype nature of the *F. rubra* population on this sub-plot. However, the genetic distance estimate relating the 13a / *F. r. commutata* population-pair is the highest of all the pairwise distances (0.185 (±0.069)). Yet, when the *F. r. commutata* 'population' is compared with the *F. r. rubra* 'population', or the other Park Grass populations for that matter, genetic distances are low.

Nonetheless, these results are likely be an artefact of the small sample set representing the *F. r. commutata* 'population'. Consequently, inferring a significant difference in the allele phenotypes displayed by ssp. *commutata* and ssp. *rubra* individuals from these values may well be misleading.

***Table 5.5*: Genetic distances between populations based on allele phenotypes at four microsatellite loci calculated using the measure of Tomiuk & Loeschcke, (Tomiuk and Loeschcke, 1996), implemented in POPDIST (Guldbrandtsen *et al.*, 2000).**

Pop	*10b*	*13a*	*13d*	*16d*	*17a*	*17d*	*Ash*	*Crc*	*Frc*	*Frg*	*Frr*
10b-11											
13a-1	0.139 (0.039)										
13d-1	0.092 (0.032)	0.089 (0.021)									
16d-36	0.074 (0.025)	0.054 (0.007)	0.032 (0.011)								
17a-1	0.101 (0.037)	0.081 (0.016)	0.068 (0.023)	0.056 (0.020)							
17d-1	0.089 (0.025)	0.102 (0.018)	0.082 (0.023)	0.040 (0.010)	0.096 (0.030)						
Ash-1	0.088 (0.019)	0.113 (0.020)	0.111 (0.010)	0.066 (0.015)	0.121 (0.031)	0.080 (0.017)					
Crc-1	0.071 (0.024)	0.065 (0.016)	0.057 (0.021)	0.039 (0.012)	0.074 (0.023)	0.054 (0.011)	0.112 (0.007)				
Frc-1	0.107 (0.040)	0.185 (0.069)	0.039 (0.012)	0.044 (0.015)	0.056 (0.020)	0.069 (0.018)	0.098 (0.021)	0.104 (0.039)			
Frg-1	0.082 (0.018)	0.059 (0.013)	0.062 (0.009)	0.051 (0.010)	0.101 (0.028)	0.066 (0.009)	0.044 (0.006)	0.065 (0.011)	0.123 (0.039)		
Frr-1	0.067 (0.024)	0.097 (0.024)	0.035 (0.011)	0.035 (0.013)	0.069 (0.026)	0.075 (0.022)	0.088 (0.019)	0.063 (0.022)	0.053 (0.019)	0.064 (0.013)	
Plch-1	0.083 (0.023)	0.064 (0.011)	0.052 (0.011)	0.028 (0.006)	0.068 (0.016)	0.057 (0.013)	0.049 (0.009)	0.048 (0.008)	0.075 (0.019)	0.053 (0.010)	0.064 (0.016)

N.B: Figures in parentheses are the jacknife estimations of standard error relating to the preceding genetic distance value. Distance values referred to in the text are highlighted in red.

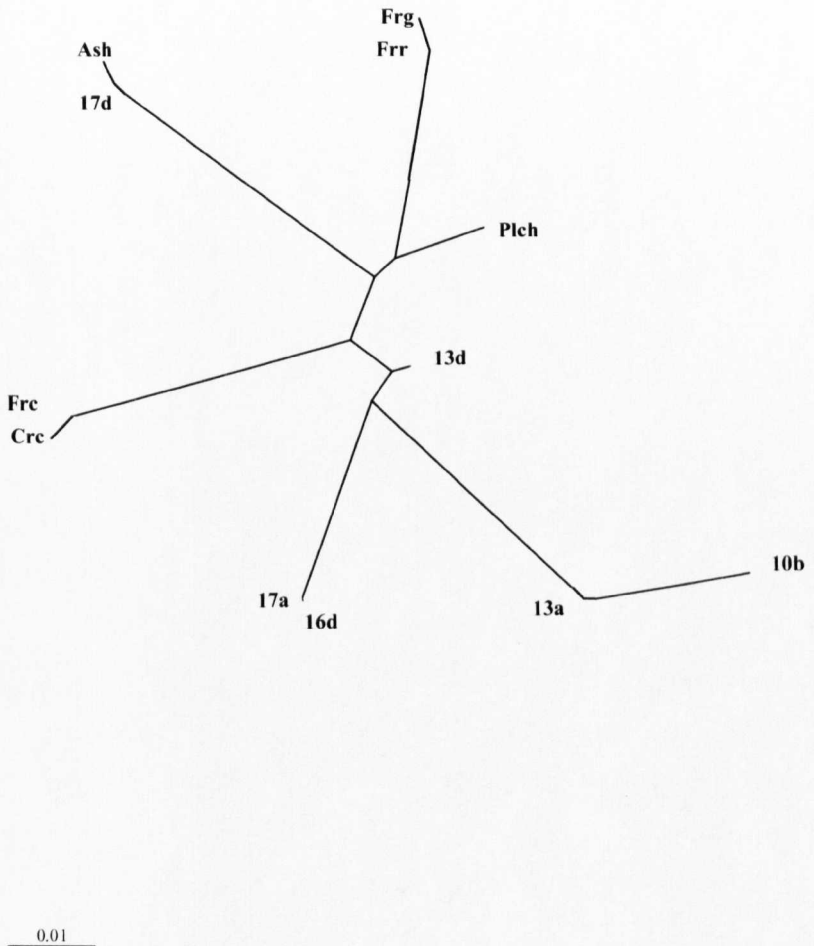


***Figure 5.4*:** Dendogram graphically representing genetic distances between populations, based on allele phenotypes at four microsatellite loci. The phenogram is based on genetic distances calculated in Popdist (Guldbrandtsen *et al.*, 2000) using the measure of Tomiuk & Loeschcke, (Tomiuk and Loeschcke), and generated using the PHYLIP package (Felsenstein, 1993).

Table 5.6 shows the physical positioning of allelic phenotypes along the transects within the Park Grass populations that were sampled. Each of the genotypes distinguished have been given a number for individual identification. None were found present on more than a single sub-plot.

Table 5.6: The physical positioning of genotypes (numerically distinguished) on the 2.5m transects along which they were sampled

Distance along transect (cm)	Genotype number					
	10b	**13a**	**13d**	**16d**	**17a**	**17d**
0	15	22	44	64	82	102
10	1	33	42	57	89	92
20	12	33	45	67	89	106
30	19	33	47	67	83	104
40	12	35	43	67	80	103
50	13	25	49	73	82	96
60	15	38	49	73	88	94
70	14	34	54	71	81	95
80	10	34	41	74	80	97
90	16	36	54	55	87	112
100	9	28	54	74	86	98
110	7	27	46	69	85	113
120	8	26	52	56	78	107
130	8	37	52	70	84	112
140	8	29	53	72	80	108
150	18	23	53	62	80	110
160	18	30	50	59	80	109
170	17	24	50	63	75	111
180	20	31	48	62	76	93
190	11	32	50	61	80	101
200	4	32	50	60	81	91
210	6	40	51	58	77	100
220	5	21	50	65	77	99
230	2	39	50	68	76	90
240	3	/	48	66	79	105

N.B: numbered genotypes highlighted in red represent known clones, as determined from allelic phenotypes generated through amplification of four microsatellite loci.

Discussion

In this study, allelic phenotypes were used to estimate the proportions of clonally and sexually reproduced individuals, which were found to differ significantly between sub-plots of the PGE. The possibility that individuals exhibiting the same allelic phenotypes in fact represent different genotypes must be considered, as this factor may be seen to bias the results described here in favour of clonal reproduction.

In view of the octaploid nature of the *F. r. rubra* genome, the fact that two individuals share several alleles at each of three nuclear loci does not necessarily indicate genetic identity. Unless each of the alleles considered is represented on the same number of chromosomes, and these chromosomes are homologous, the individuals will not represent the same genotype. Indeed, differences in allelic dosage are likely to account for the representation of a single allelic phenotype by an array of genotypes.

Consequently, lengthy consideration was given to the design of a model capable of estimating the probability that two individuals, exhibiting the same allelic phenotype, have different genotypes. An attempt was made by Parks & Werth, (Parks and Werth), to determine the probability of individuals with identical genotypes representing the same genet. However, the model they suggest obviously requires knowledge of allelic dosage, precluding its application to allelic phenotype data and species of high ploidies. It also makes assumptions of no somatic mutation, random mating or linkage among loci. These assumptions may render the model unrealistic and yet would apply to any similar procedure that might be suggested here.

Perhaps the more relevant question is, 'what is the likelihood that two individuals with the same allelic phenotype are not identical by descent?' This likelihood becomes much smaller as the number of allelic phenotypes rises. Given the large

number of alleles found at each of the nuclear loci, the potential number of allelic phenotypes that might be expressed within a population is enormous.

In this study no allelic phenotypes were found shared by individuals from different populations and those that were shared within a sub-plot were a maximum of 1.5m apart. Consequently, it is far more likely that sharing of allelic phenotypes by individuals in these investigations is the result of clonal reproduction rather than the unlikely convergence upon a single allelic phenotype by two distinct genotypes within the immediate vicinity of one and other.

From the estimated ratio of clonally to sexually reproduced individuals it is possible to infer the selective pressures on recruitment mode of *F. rubra* within each population; selection for sexual recruitment appears to be greater within the populations of sub-plots 10b, 16d, 13a and 17d, whilst selection for clonal recruitment appears to be greater within the populations of sub-plots 17a and 13d.

The mode of reproduction appears to be correlated with rate of environmental change in four of the six populations investigated in this study. Sub-plots 10b and 16d have both witnessed significantly elevated rates of change and this appears to be reflected in the observed bias toward sexual recruitment on each.

It was determined in Chapter 2 that the inferred rates of environmental change over the past 140 years have been noticeably lower on sub-plots 17a, 17d, 13a and 13d than on the two sub-plots discussed above. Indeed, the Mean Character Difference (MCD) metric suggests the communities of these four sub-plots have been relatively stable, undergoing minimal change comparable to that experienced by the community of sub-plot 3d which acts as a control for the PGE itself.

However, of these four populations, only those of sub-plots 17a and 13d fit with our expectations of selection favouring clonal recruitment under stable environmental

conditions. The remaining two populations, those of sub-plots 17d and 13a, appear to contradict our expectations.

This might be explained for the population of sub-plot 17d by the observed disagreement in results from the statistical methods applied in Chapter 2. As already mentioned, the (MCD) metric suggests minimal community change on sub-plot 17d. Yet the results of the Detrended Correspondence Analyses (DCA) suggest this same community has witnessed moderate rates of change since the mid 1970's that were three to four times greater than that experienced by control sub-plot 3d. If the estimated rates of change on sub-plot 17d provided by the DCA are more accurate than those suggested by the MCD metric, then this may explain the observed selection bias toward sexual recruitment within this population.

However, such an explanation does not exist for the unexpected bias toward sexual recruitment on sub-plot 13a, since the results of both the DCA and MCD metric suggest environmental stability on this sub-plot since the late 1940's. Nonetheless, the *F. rubra* population of sub-plot 13a was identified in Chapter 4 as mixed-cytotype. Consequently, a bias toward sexual reproduction within this population should not be inferred since the sample set might represent an admixture of two potentially reproductively isolated sub-species displaying differing strategies of clonal spread.

If we discount the mixed cytotype population, we find that four of the five remaining populations display a correlation between rate of environmental change and mode of reproduction in *Festuca rubra* ssp. *rubra*. Furthermore, the significantly greater proportion of sexual to clonal genotypes within the unpredictable environments of sub-plots 10b and 16d suggests a selection bias toward sexual recruitment and a limited response of generalist genotypes to environmental change.

Skalova *et al.*, (Skalova *et al.*), identified *F. rubra* clones exhibiting high levels of plasticity in response to environmental variability. However, the studies were based on populations within fine-scale heterogeneous environments of mountain grasslands in the Czech Republic. The populations of sub-plots 10b and 16d, on the other hand, whilst likely to exhibit the levels of spatial heterogeneity common to grasslands in general, have been identified here as having experienced *extreme* temporal heterogeneity. Furthermore, whilst plasticity in traits such as rhizome length and production may facilitate the escape of some genets from unfavourable environments, if temporal heterogeneity is particularly high, then clonal spread may not offer a rapid enough means of dispersal or colonisation to maintain population numbers.

In plants, pollen and seeds often differ from the structures of vegetative propagation (rhizomes, stolons and bulbils) in their dispersal abilities (Cleplitis, 2001), and this trend is very likely to hold true for the wind-pollinated *Festuca rubra*. Indeed, physical movement of *F. rubra* ramets in mountain grassland habitats of the Czech Republic was estimated at ~1cm per year (Herben *et al.*, 1993). Hence, this is likely to represent an extremely limited means of dispersal compared to seed and pollen movement.

The elevated levels of temporal heterogeneity experienced by the populations of sub-plots 10b and 16d may have been translated into increased rates of genet mortality and an associated increase in recruitment opportunities. However, owing to the limited dispersal abilities of *F. rubra* genets, colonisation of those patches available for recruitment via vegetative propagation is likely to have been prohibited. The physical distance between such patches and any potentially well-adapted genotypes from other localities would simply be too great. However, such restrictions associated with dispersal abilities are not likely to affect seed recruitment. Hence, it is possible to see

how the observed increases in sexual recruitment on sub-plots that have experienced elevated rates of environmental change may have occurred.

On those sub-plots whose populations have experienced less temporal variation, and in which clonal recruitment appears to be favoured, genet diversity has nonetheless remained high. The present study has not found these stable environments to be dominated by one or two genets, which would imply competitive exclusion of others or differential success of individual genets under different environmental conditions (Suzuki *et al.*, 1999). Rather, these populations are each represented by a large number of genets.

The absence of extreme temporal variation in these otherwise fine-scale spatially heterogeneous environments may have favoured the clonal recruitment of plastic generalist genotypes. Under such a scenario opportunities for seedling recruitment are likely to be uncommon and this may account for the observed ratios of sexual to clonal genotypes within these sub-plots.

As already discussed, deviation of the sub-plot 17d population from our expectations of clonal recruitment may be due to inaccuracy in our estimated rate of environmental change on this sub-plot. Of the six communities investigated, only the results from that of sub-plot 17d proved so contradictory in the analyses of community change; the MCD metric suggested a low rate of environmental change whereas the DCA suggested a more intermediate rate.

Similar contradictions were obtained from the Total Proportional Dissimilarity and City Block metrics employed in Chapter 2, though these results were discounted owing to the unsuitability of the metrics to our particular data sets.

The composition data from sub-plot 17d seems to contain values which have an adverse effect on at least some of the statistical analyses employed (possibly the large

number of rare species). However, identifying which of the two favoured statistical methods is overly sensitive to the 17d composition data is difficult without further extensive analysis. Hence, the possibility exists that this sub-plot has in fact experienced elevated rates of environmental change and that the observed number of genotypes is in line with expectations.

The aims of this Chapter in determining the relative ratios of clonal to sexual recruitment required non-random sampling methods within each of the Park Grass populations. This is likely to render associated estimates of pairwise population genetic distances inaccurate since the samples representing each sub-plot (collected at 10cm intervals along 2.5m transects) are expected to provide unrealistic representations of the diversity present within each population. Similarly, estimates of within-population diversity generated from these sample sets are likely to be lower than the true values.

Nonetheless, it is interesting to notice that the Shannon-Weaver (S-W) values of within population phenotype diversity associated with sub-plots 10b and 17d are in a comparable range with that of the Ashridge population and higher than that of the Cricklade population. Only the sub-plot 13a population has an S-W phenotype diversity value noticeably lower than those of populations external to the PGE. This may reflect the strong bias toward clonal recruitment and the relatively low number of genotypes within this population. Alternatively, it may be related to the observed genetic differentiation of the population from others on the Park Grass meadow, as illustrated by the genetic distance data of Table 5.5. Such differentiation may have been a consequence of strong selective pressures which can also purge populations of their genetic diversity.

The fact that samples taken from within each sub-plot population were physically separated by between just 10cm and 240cm, whilst those of the other populations outside the PGE were separated by anything from 10m to 200m, suggests comparatively high levels of standing genetic diversity within *Festuca rubra* populations of the Park Grass meadow.

We can only speculate as to the source of such population level diversity, though in the past it has been noted that *Festuca rubra* exhibits high levels of genetic variation (Grime *et al.*, 1988). The observed phenotypic diversities of the Park Grass populations and their high genetic distances from one another (see the genetic distance matrix of Table 5.5) may be the result of the array of selective pressures imposed upon them, through the application of various fertilizer treatments, and an associated rapid divergence of the populations coincidental with local adaptation. Table 5.6 shows the physical positioning of allelic phenotypes along the sub-plot transects from which they were sampled. From the table it is possible to see how the ratio of clonal to sexual recruitment inferred for each population might alter if more than a single transect was sampled from each sub-plot; if we look at the occurrence of genotypes along the transect of sub-plot 17a we can see that genotype '80' occurs 6 times. Assuming that a clone will spread in a radially symmetrical fashion, then one might be able to imagine how the random positioning of a transect might result in its skimming the edge of a large clone in the same way that a tangent skims the edge of a curved line.

Our detection of genotype "80" a total of six times at positions of up to 1.5m apart may reflect the transect skimming the edge of a very large clone. If this is the case then alternative positioning of the transect, perhaps through the middle of the clone, might have given a result of even stronger clonal recruitment within this population.

Of course, this argument can also be used to suggest underestimation of sexual recruitment, but the point remains that given greater time and resources, more reliable estimates of clonal and sexual recruitment might be made.

In this Chapter microsatellite markers have been identified which provide a reliable, high resolution means of distinguishing even closely related individuals of *Festuca rubra*. A potential correlation between rate of environmental change and the observed mode of reproduction in populations of ssp. *rubra* has also been identified. This may be a consequence of the differing dispersal abilities afforded to individuals through vegetative propagation and seed production; in an unpredictable environment, the plasticity displayed by individuals in response to varying environmental conditions may be limited in comparison to the actual range of environmental conditions encountered. Similarly, where environmental change is both rapid and extreme, clonal spread may not be fast enough to facilitate escape to more suitable patches. Sexual reproduction, on the other hand, allows for the rapid generation of novel genotypes, a proportion of which may be well suited to any divergent environmental conditions encountered. Seeds are also capable of wider dispersal to patches within which recruitment opportunities exist.

Differing dispersal abilities of seeds and vegetative propagules may account for the maintenance of a balance between reproductive modes in sexual/clonal species (Cleplitis, 2001), and this may be true of *Festuca rubra* on Park Grass.

Further replications of the investigations described here are needed to confirm that the observed correlation is not a chance artefact of the small number of populations investigated.

Chapter 6

Conclusions

The primary objective of this study has been to test the hypothesis that mode of reproduction observed in populations of *Festuca rubra* is correlated with rate of environmental change. However, discovery that the species under investigation is represented by two sub-species on the Park Grass meadow necessitated revision and expansion of the original hypothesis. Consequently, two hypotheses were set out which relate to the primary objective. The first hypothesis states: *The mode of recruitment in both F. r. ssp. rubra and ssp. commutata varies between sub-plots and is correlated with the amount of environmental variation experienced by a population. Greater environmental variation should result in more recruitment via sex and less from clonal reproduction.*

The second hypothesis was written with consideration of *F. r. commutata*'s inability to reproduce clonally, and states: *Since F. r. commutata has no rhizomes and reproduces sexually, its abundance should be positively correlated with the amount of environmental variation experienced by a population.*

The secondary objective of these studies has been to determine whether the *Festuca rubra* populations investigated on sub-plots of the Park Grass meadow reflect ones' expectations of minority cytotype exclusion. Consequently, a third and final hypothesis was drawn which states: *Festuca rubra will be represented by single cytotype populations on the separate sub-plots of the PGE.*

The three hypotheses will be addressed individually.

Overview: As explained in Chapter 1, meiosis and sexual reproduction carries a 50% cost when compared to the production of genetically identical progeny by, for instance, vegetative propagation (Maynard Smith, 1978; Williams, 1975). Yet, the reproductive advantage of sex, which must presumably exist owing to its ubiquitous

nature among species of the Earths' biota, has still to be accounted for. Over a century ago August Wiesmann, speculated that sexual reproduction facilitates evolutionary adaptation by increasing a population's genetic variation. This hypothesis has been supported by contemporary research on sexual and asexual yeast strains (Goddard *et al.*, 2005). Yet, in yeast there is no male-female distinction and hence understanding the advantage of sex in light of its' two-fold cost amongst plants and animals is far from understood (Hoekstra, 2005).

Festuca rubra has been used in this study as a model organism to investigate factors likely to promote sexual recombination; it reproduces clonally, via vegetative propagation, and sexually via seed reproduction. Clones appear to have localized distributions, though may reach considerable sizes (clones exhibiting the same allelic phenotype were found 1.5m apart in this study), and have been estimated to persist for extremely long periods of time of up to 1000 years (Harberd, 1961)!

Similarly, the Park Grass meadow has provided a rare opportunity for studying the consequences of contrasting rates of environmental change on recruitment in this species. Other studies have focussed on variables such as interspecific competition (Rautiainen *et al.*, 2004), population density (van Kleunen *et al.*, 2001) and life history characteristics (van Kleunen *et al.*, 2002), though none have provided a compelling explanation for the paradox of sex.

The investigations described here, on the other hand, have made use of 150 years of species composition data to investigate the effects of rate of environmental change – a variable that few other researchers have had the opportunity to consider.

In Chapter 2 sensitive statistical metrics and analyses have been employed to identify populations that have experienced widely contrasting rates of environmental change. In Chapter 3 the potential for hybridisation and introgression has been highlighted

between *F. rubra* ssp. *rubra* and ssp. *commutata*, a sub-species that was previously unrecorded on the Park Grass meadow. Of the populations under scrutiny, those that are monospecific for ssp. *rubra* have been identified in Chapter 4, as has the single population containing a mixture of both ssp. *rubra* and ssp. *commutata* individuals. Finally, in Chapter 5, highly variable molecular markers have been identified, capable of distinguishing even closely related genotypes, facilitating estimation of the relative frequencies of clonal and sexual recruitment within the study populations.

Discussion related to Hypothesis 1:

The low frequency representation of ssp. *commutata*, found in only one of the study populations, prevents any suggestions from being made relating to the mode of reproduction observed in this sub-species under differing circumstances of environmental change. However, a potential correlation has been detected from amongst the five populations exclusively represented by ssp. *rubra* in which the recruitment of sexual propagules appears to be greater, relative to clonal recruitment, within populations that have experienced elevated rates of environmental change. Alternatively, the observed variation in recruitment strategy amongst populations may be related to one or more factors other than rate of environmental change. However, consideration has been given to the treatments applied to each sub-plot, and their associated soil chemistries, and no other correlation is evident.

The Strawberry-Coral model of sexual and asexual reproduction: In his book 'Sex and Evolution', Williams, (Williams), suggested numerous models to account for prevalence of the "seemingly maladaptive character of sexual reproduction" amongst different plants and animals. Of the models described, the Strawberry-Coral model

appears applicable to ssp. *rubra*, since the potential correlation between mode of reproduction and rate of environmental change observed in this sub-species may reflect disparity in the dispersal abilities of its clonal and sexual propagules; vegetative propagation may enable persistence of well-adapted genotypes in stable environments where spatial and temporal heterogeneity fluctuate within the limits of genotypic plasticities. Conversely, sexual reproduction may allow for colonisation of isolated patches, through the production and distribution of seeds, and the perpetuation of genes within environments in which extremely adverse conditions repeatedly occur.

In describing the Strawberry-Coral model Williams, (Williams, 1975), wrote;

"*Evolutionary success will require vagile propagules capable of establishing themselves beyond clonal boundaries. Since these boundaries define the region of adequacy of a clone's genotype, it is necessary that these clones be sexually reproduced*".

The model further suggests that an increase in the density of clones representing the same genotype will result in elevated levels of inter-clone competition and an associated increase in sexual reproduction. Since it is the *consequences* of increased densities of genetically identical clones (i.e. diminishing returns from investments into clonal propagation) that are responsible for associated increases in sexual reproduction, it is possible to envisage how other stresses that bring about the same consequences are likely to result in a similar increase in sexual reproduction.

Under the Strawberry-Coral model, elevated temporal heterogeneity in environmental conditions amongst the populations investigated here might be regarded as a stress that is likely to contribute to a decrease in reproductive output via vegetative propagation. Indeed, the number of clonal genotypes identified were significantly

fewer on those sub-plots of the PGE that have undergone elevated rates of change than on two of the three sub-plots that have histories of environmental stability. This suggests a decrease in clonal recruitment associated with increasing levels of environmental stress. Such dynamics in sexual *versus* clonal recruitment appear to be similar to those reported for wild rice, *Oryza rufipogon*, whose populations have been shown to display elevated rates of sexual recruitment under circumstances of external disturbance or seasonal drought (Xie *et al.*, 2001).

The degree of environmental stress experienced by a genotype is likely to be reflected in its fitness, such that fitness is reduced as levels of the environmental stress, (in this case rate of environmental change), are increased. Consequently, the findings of this study may be seen to suggest that *Festuca rubra rubra* displays fitness-associated recombination, because recombination is negatively correlated with the fitness of genotypes.

Distinguishing reproductive allocation from realised recruitment: There are two possible approaches to the primary question of this thesis; does environmental variation favour sex in *Festuca rubra* ssp. *rubra* and ssp. *commutata*? The first approach, which has been adopted in this and other studies (Graser *et al.*, 1996), is to look at the frequency of individual genotypes and realised recruitment rates within populations as a means of determining the relative success of clonal versus sexual reproduction. It does assume that no somatic mutations have occurred between generations of clonal individuals, but previous studies have found no evidence of such genetic divergence amongst clone-mates of polyploid apomictic dandelions (van der Hulst *et al.*, 2000). An alternative approach might be to measure the allocation to both sexual and clonal reproduction in individual plants occupying different sub-plots.

However, in practice this cannot be accomplished *in situ*, requiring instead common garden experiments that could not be related to specific sub-plots. Of course, ideally both approaches would have been adopted, though the second approach may still be the subject of future investigations. Nonetheless, a bias toward sexual recruitment within rapidly changing environments suggests a selective advantage to sexual reproduction. This would underpin both the allocation by clones to sexual reproduction associated with localised recruitment, and sexual recruitment from different localities.

Consequently, the observations made here of a potential correlation between the advantages of sexual reproduction and rate of environmental change may be fundamental to the evolution and maintenance of sexual reproduction.

Limitations of clonal propagation under the Strawberry-Coral model: The success of a clonal genotype in a heterogeneous environment is limited by its rate of clonal spread and the degree of plasticity it is capable of displaying in response to environmental conditions (Cain, 1994; Oborny, 1994). Consequently, under rapidly changing and grossly adverse environmental conditions, such limitations may become apparent; the clone may experience a heightened risk of mortality and significantly reduced rates of vegetative propagation. It may also display an inadequate rate of escape to more favourable environments.

Advantages of sexual reproduction under the Strawberry-Coral model: Recombination, however, offers an alternative means of perpetuating genes through to future generations. Even in circumstances where environmental conditions are particularly favourable to a well-adapted clone, mortality will eventually occur, either

as a result of environmental change or competitive exclusion. The physical boundaries of a clone represent the locality within which it is capable of out-competing other genotypes (Williams, 1975). Other patches within which it may also be well-adapted may exist, but are unlikely to be in close enough proximity to allow escape. Consequently, whether a clone inhabits a temporally stable environment, or an unpredictable environment, sexual reproduction offers a means of assuring representation of an individual's genes within populations of future generations.

Recruitment of *Festuca rubra* on Park Grass relative to other natural and semi-natural systems: The sub-plots identified in this study that have experienced elevated rates of change are likely to represent a relatively small proportion of natural and semi-natural grassland habitats compared to those sub-plots with histories of comparative stability. The changes in species assemblages and soil chemistries on the high-change sub-plots have been brought about by considerable and repeated human intervention. Comparable rates of environmental change over such extensive time periods, perhaps through a series of chance events, are likely to be uncommon within more natural circumstances. Consequently, it might be expected that any potential bias toward sexual recruitment associated with episodes of rapid environmental change occur infrequently in natural and semi-natural grassland systems.

The observations made here on those sub-plots with histories of relative stability are more likely to reflect the norm. Their levels of temporal and spatial heterogeneity are expected to be more consistent with those of natural and semi-natural systems, such as the grasslands studied by Skalova *et al.*, (Skalova *et al.*, 1997), in the Krkonose mountains of the Czech Republic.

Discussion related to Hypothesis 2: There appears to be no correlation between rate of environmental change and abundance of ssp. *commutata*. *F. r.* ssp. *commutata* is absent from five of the six sub-plots investigated even though the plant communities therein have experienced widely contrasting rates of change. Only the relatively stable sub-plot 13a contains ssp. *commutata*, though at low frequency, and hence it is not possible to draw any conclusions. The second hypothesis is therefore rejected, suggesting that abundance of ssp. *commutata* is instead governed by one or more ecological variables not considered here.

Discussion related to Hypothesis 3: Due to the detection of a mixed cytotype population on sub-plot 13a the third hypothesis of this investigation is also rejected. The presence of both ssp. *rubra* and ssp. *commutata* in this population contradicts the theoretical predictions on frequency dependent selection (Levin, 1975). The fact that only one of the six populations investigated is of mixed cytotype suggests that ecological sorting within the habitat mosaic of the Park Grass meadow has facilitated the overcoming of minority cytotype exclusion by ssp. *commutata*, rather than a reproductive isolating mechanism (the consequences of which we might expect to have seen in terms of a higher observed frequency of mixed cytotype populations). However, ecological sorting does not necessarily occur to the exclusion of other environmentally independent processes, and it may be that a combination of both ecologically dependent and independent factors within the sub-plot 13a population has facilitated coexistence of the two sub-species. Yet, with consideration given to the rejection of the second hypothesis (discussed above), one ecological variable that we can now confidently suggest as being unlikely to contribute to the coexistence of the sub-species is rate of environmental change.

Further work allowing for a more extensive survey of cytotype frequencies and distributions across the numerous sub-plots of the PGE would help in identifying the specific ecological factors of consequence to the maintenance of mixed cytotype populations of *F. rubra*. The wide range of soil chemistries and community structures exhibited on the various sub-plots offers a worthwhile opportunity to identify the specific variables affecting the outcome of frequency dependent selection between these sub-species.

Final Conclusions:

The observed bias toward clonal recruitment on sub-plots with histories of stability may represent a generalist strategy of reproduction in *Festuca rubra* in line with expectations for systems exhibiting fine-scale spatial variability (van Tienderen, 1991). Under low rates of environmental change, temporal variations in conditions are likely to be within the limits of response through individual plasticity. In such circumstances, an increased allocation to clonal reproduction provides a means of growth and spread, free of the costs associated with sexual recombination.

The allocation of resources to sexual reproduction may be justified under either of two scenarios. In view of the inevitable mortality of clonal genotypes and the limited means of dispersal and escape exhibited by clones, should conditions be encountered that are outside of the limits of plastic response by individuals, recombination provides a necessary means of genetic dispersal, both temporally (i.e. amongst different genotypes of future generations) and spatially (i.e. across physical distances that could not be crossed by means of clonal propagation). Whether such allocation to sexual reproduction is an immediate, short-term response to unfavourable

environmental conditions encountered by clones, perhaps in the form of fitness-associated recombination, or a sustained, long-term strategy favoured by selection as a consequence of the inevitable mortality of every clone, cannot be differentiated in this study. Further investigations into the reproductive allocation of genotypes with associated estimations of realised recruitment rates of clonal and sexual propagules amongst populations exhibiting contrasting histories of change would be of great value in determining whether sexual reproduction holds a long or short-term selective advantage at the population level. Similarly, it would be desirable to increase the sample size of populations examined in this study, so that meaningful regression analyses may be applied to the data. This would make it possible to test whether the correlation, as suggested by the existing data, between rate of environmental change and mode of reproduction selected for in *F. rubra* ssp. *rubra*, is significant.

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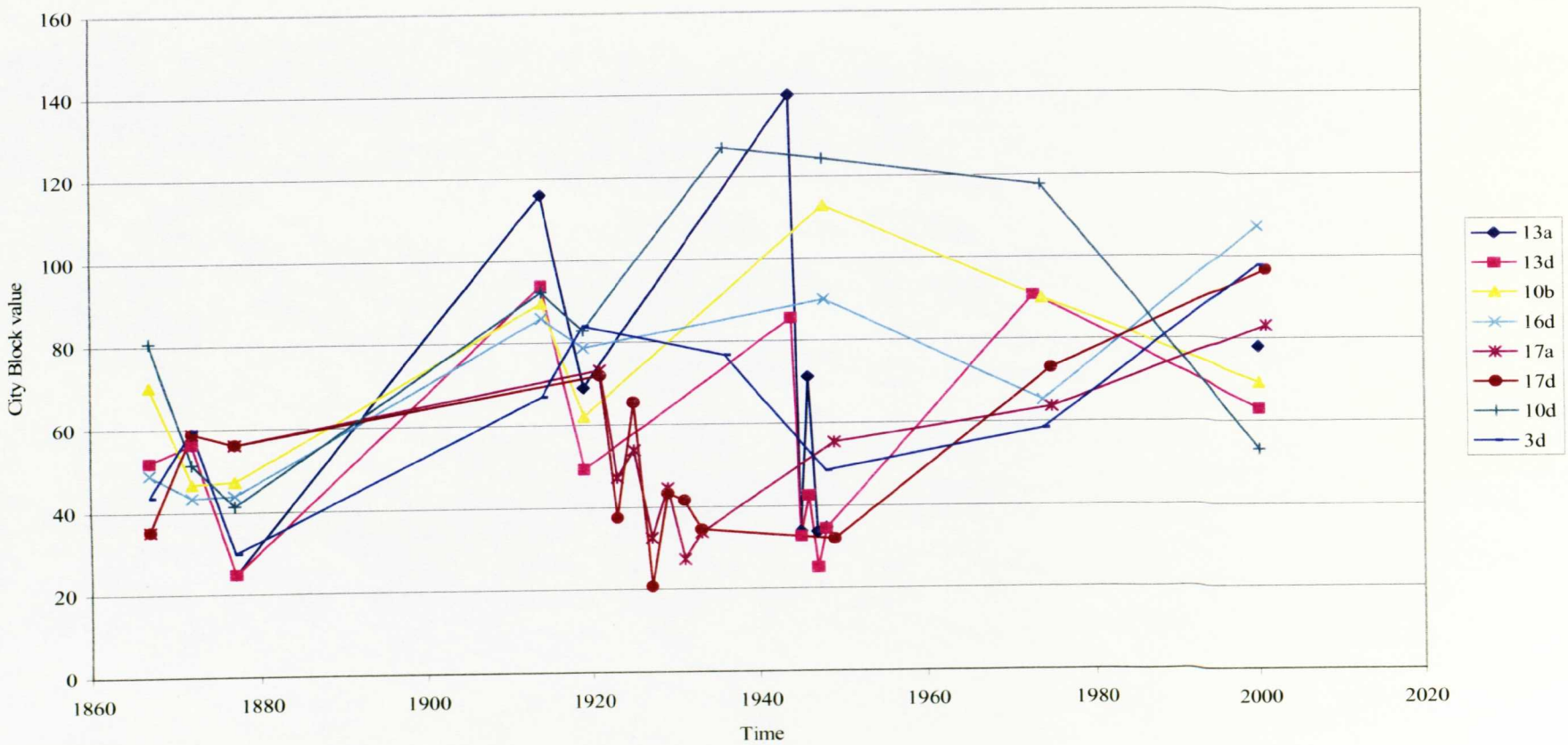
Appendices

***Appendix 1*: Names and abbreviations of species considered in the statistical analyses of changes in plant communities described in Chapter 2.**

NAME	Code	NAME	Code
Achillea millefolium	A. mi	*Knautia arvensis*	K. ar
Achillea eupatoria	A. eu	*Lathyrus pratensis*	L. pr
Agrostis capillaris		*Leontodon hispidus*	L. hi
Agrostis tenuis	A. cap	*Linum catharticum*	L. cat
Agrostis vulgaris		*Lolium perenne*	L. pe
Aira caespitose	A. cae	*Lotus corniculatus*	L. co
Deschampsia caespitosa		*Luzula campestris*	L. cam
Ajuga reptans	A. re	Mosses	Moss
Alopecurus pratensis	A. pr	*Ononis arvensis*	O. ar
Anthoxanthum odoratum	A. od	*Ononis repens*	O. re
Anthriscus sylvestris	A. sy	*Ophioglossum vulgatum*	O.vu
Arrhenatherum avenaceum	A. av	*Phleum pratense*	p. pr
Arrhenatherum elatius	A. el	*Pimpinella saxifraga*	P.sa
Avena flavescens	A. fla	*Plantago lanceolata*	P. la
Trisetum flavescens		*Poa pratensis*	Poa. pr
Avena pubescens	A. pu	*Poa trivialis*	P.tr
Briza media	B. me	*Potentilla reptans*	P. re
Bromus hordaceus	B. mo	*Potentilla sterilis*	
Bromus mollis		*Prunella vulgaris*	P. vu
Carex caryophyllea	C. car	*Quercus robur*	Q. ro
Carex flacca	C. fl	*Ranunculus acris*	Ran. Sp
Centaurea nigra	C. ni	*Ranunculus bulbosus*	
Cerastium fontanum		*Rumex acetosa*	R. ace
Cerastium holosteoides	C. fo	*Rumex crispus*	R. cri
Cerastium triviale		*Poterium sanguisorba*	s. mi
Cerastium vulgatum		*Sanguisorba minor*	
Chrysanthemum leucanthemum	C. le	*Scabiosa arvensis*	S. ar
Conopodium denudatum	C. de	*Senecio jacobaea*	S. ja
Conopodium majus		*Stellaria graminea*	S. gr
Crepis capillaris	C. cap	*Taraxacum officinale*	T. of
Cynosurus cristatus	C. cr	*Taraxacum vulgare*	
Dactylis glomerata	D. gl	*Thymus serphyllum*	T. se
Festuca ovina		*Tragopogon pratensis*	Tra. Pr
Festuca pratensis	F. ru	*Trifolium pratense*	Tri. pr
Festuca rubra		*Trifolium repens*	T. re
Galium verum	G. ve	*Urtica dioica*	U. di
Heracleum sphondylium	H. sp	*Veronica chamaedrys*	V. ch
Hieracium pilosella	H. pi	*Vicia sativa*	V. sa
Holcus lanatus	H. la	*Vicia sepium*	V. se
Hypochoeris radicata	H. ra		

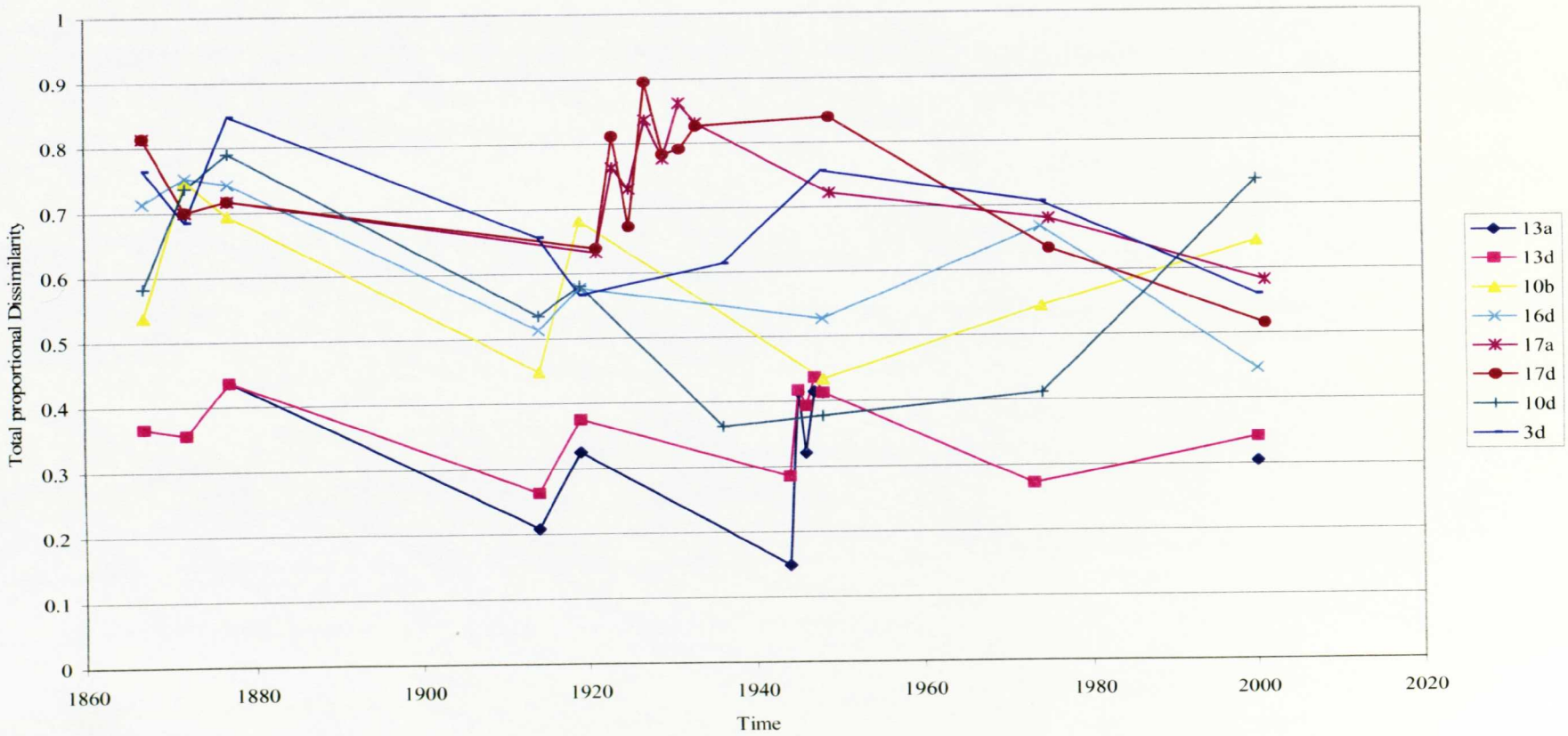
***Appendix 2*:**

Change in City Block values on all plots between 1862 and 2000



***Appendix 3*:**

Change in Total proportional Dissimilarity on all plots between 1862 and 2000



***Appendix 4*: 158 base pair alignment of ITS 1 region sequences generated in this study.**

```
Position    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                 5          15         25         35         45         55         65         75
Frr5-c-1    TGCCTAACCC GGGGACGCGG CTGGCTT-GC TGGCCGCACC TTGTGTTGCA ATGCTATATA ATCCACACGA CTCTCGGCAA
Frr2-b-1    ----------*********-** ********** ********** ********** ********** ********** **********
Frr1-b-1    ********** ********** *******-** ********** ********** ********** ********** **********
Frc1-b-1    ********** ********** *******-** ********** ********** ********** ********** **********
Frr5-b-1    ********** ********** *******-** ********** ********** ********** ********** **********
Frc2-a-4    ********** ********** *******T** ********** ********** ********** ********** **********
Frr8-a-2    ********** ********** *******-** ********** ***G****** ********** ********** **********
Frr4-c-1    *********- ********** *A*****-** ********** ***G****** ********** ****G***** **********
Frr4-d-1    ----------*** ******* *******-** ********** ***G****** ********** ********** **********
Frc4-11-8   ----------*** ******* *******-** ********** ***G****** ********** ********** **********
Frr2-a-4    *********- ********** *******-** ********** ***G****** ********** ********** **********
Frr4-a-1    *********- ********** *******-** ********** ***G****** ********** ********** **********
Frr5-a-2    *********- ********** *******-** ********** ***G****** ********** ********** **********
Frr6-a-1    *********- ********** *******-** ********** ***G****** ********** ********** **********
Frr3-a-4    ********** ********** *******-** ********** ***G****** ********** ********** **********
Frr7-a-3    ********** ********** *******-** ********** ***G****** ********** ********** **********
Frr1-a-4    ********** ********** *******-** ********** ***G****** ********** ********** **********
Frc3-b-1    ********** ********** *******-** ********** ***G****** ********** ********** **********
Frc1-a-3    ********** ********** *******-** *******N** ***G****** ********** ********** **********
Frc3-a-1    ********** ********** *******-** ********** ***G****** ********** ********** **********
Frc1-c-1    *********- ********** *******-** ********** *A*G****** **A******* ********** **********
Frr7-b-1    ********** ********** *******-** ********** *A*G****** ********** ********** **********
Frr4-b-1    ********** ********** *******-** ********** *C*G****** ********** ********** **********
```

```
Position    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|...
                85         95        105        115        125        135        145        155
Frr5-c-1    CGGATATCTC GGCTCTCGCA TCGATGAAGA ACGTAGCGAA ATGCGATACC TGGTGTGAAT TGCAGAATCC CGCGGAAC
Frr2-b-1    ********** ********** ********** ********** ********** ********** ********** ****-***
Frr1-b-1    ********** ********** ********** ********** ********** ********** ********** ****-***
Frc1-b-1    ********** ********** ********** ********** ********** ********** ********** ****-***
Frr5-b-1    ********** ********** ********** ********** ********** ********** ********** ****-***
Frc2-a-4    *********N ********** ********** ********** ********** ********** ********** ****-***
Frr8-a-2    ********** ********** ********** ********** ********** ********** ********** ****-***
Frr4-c-1    ********** ********** ********** ********** ********** ********** ********** ****-***
Frr4-d-1    ********** ********** ********** ********** ********** ********** ********** ****-***
Frc4-11-8   ********** ********** ********** ********** ********** ********** ********** ****-***
Frr2-a-4    ********** ********** ********** ********** ********** ********** ********** ****-***
Frr4-a-1    ********** ********** ********** ********** ********** ********** ********** ****-***
Frr5-a-2    ********** ********** ********** ********** ********** ********** ********** ****-***
Frr6-a-1    ********** ********** ********** ********** ********** ********** ********** ****-***
Frr3-a-4    ********** ********** ********** ********** ********** ********** ********** ****-***
Frr7-a-3    ********** ********** ********** ********** ********** ********** **N******* ****-***
Frr1-a-4    ********** ********** ********** ********** ****N***** ********** ********** ****-***
Frc3-b-1    ********** ********** **N******* ********** ********** ********** ********** ****-***
Frc1-a-3    ********** ********** ********** ********** ********** ********** ********** ****-***
Frc3-a-1    ********** **N******* ********** ********** ********** ********** ********** ****-***
Frc1-c-1    ********** ********** ********** ********** ********** ********** ********** ****-***
Frr7-b-1    ********** ********** ********** ********** ********** ********** ********** ****-***
Frr4-b-1    ********** ********** ********** ********** ********** ********** ********** ****-***
```

Appendix 5: Methodology for the estimation of plant nuclear DNA content via flow cytometric analysis.

Accuracy in cytometric analysis of nuclear DNA contents is vital if estimations of species nuclear genome sizes and the identification of intercytotype hybrids are to be reliable. Consequently, this methodology for the estimation of plant nuclear DNA content via flow cytometry aims to be transparent in both its purpose and practical application.

Methods relating to sample collection, storage and the subsequent isolation and staining of cell nuclei are described in the methods section of Chapter 4. More technical information relating to instrument precision and accuracy, use of an appropriate internal DNA standard, the requirement for replication in DNA content estimation and subsequent statistical analysis of resulting data are provided below.

Checking instrument accuracy: Instrument precision in DNA content analysis is critical since small differences in DNA content may prove biologically significant. This fact is especially relevant to our study since there is relatively little separation in the reported nuclear DNA content ranges of the two sub-species considered.

With respect to flow cytometry, linearity in the relationship between channel numbers (effectively positions on the x-axis) of a sample's 2C peak (representing individual diploid nuclei) and its' 4C peak (representing doublets of diploid nuclei, or nuclei in the synthesis phase of mitosis) is a necessity. This means that the channel number of a 4C peak should be twice that of a 2C peak. If the instrument is unable to provide a linear response it will not be possible to accurately determine the DNA content of unknown samples. Instrument precision is further monitored by measuring the 'Coefficient of Variation' (CV) of the 2C peak of a test sample.

Instrument linearity checks were carried out at the start of each day's work and the values routinely logged. The linearity check procedure was carried out using Trout Erythrocyte Nuclei, (TEN) (Partec; Germany). Trout erythrocyte nuclei are taken from what are not true cycling cells. They do not have an S-phase (the DNA synthesis phase of mitosis) fraction and hence they are a useful tool for assessing instrument resolution. TEN are also intentionally formulated to aggregate with one another such that, along with single nuclei, there are also nuclei-doublets, triplets, quadruplets and so on. The consequential ratio of quadruplet:triplet:doublet:singlet peak channel numbers allows for evaluation of instrument linearity.

TEN were run through the instrument and the 'Gain' subsequently adjusted such that the positioning of the 2C peak lay at channel 200(±5) on the x-axis. The flow rate was adjusted to ~15 nuclei per second so the cytometer did not mistake aggregates of two, three or four nuclei as single nuclei. As with all samples, after running the TEN through the machine for ~one minute, analysis was stopped and re-started so that data collection only took place once the cytometer had stabilised. TEN were then run through the instrument until 5,000 nuclei had been detected. The mean channel numbers of the 2C and 4C peaks were noted and linearity calculated using the following formula:

Instrument linearity = mean channel no. of 4C peak / mean channel no. of 2C peak

Instrument linearity was considered reliable if found to lie between 1.95 and 2.05. The percentage 'Coefficient of Variance' (CV) of the TEN 2C peak was also checked. As is described in the Becton Dickinson user guide for the FACScalibur flow cytometer, if the CV was found to be $\leq 2.0\%$, a high level of confidence could be placed in the

instruments' accuracy. If the CV was found to be $\leq$ 3.5%, this could be seen to indicate that cytometer performance was still good, though one or two further replicate runs were advisable to ensure accuracy of results. However, if the percentage CV rose above 3.5%, steps were taken to improve the instruments performance before continuing further. Again, the CV values obtained prior to each day's analyses were recorded as proof of the instrument's accuracy.

Running a DNA standard and samples of unknown DNA content: When carrying out DNA content analysis via flow cytometry, it is necessary to run an appropriate 'standard' sample of known nuclear DNA content. This serves as a benchmark against which calculations of DNA content of unknown samples can be made. The procedure is carried out by adjusting the positioning of the standard samples' 2C peak to channel 200 on the x-axis. Channel 200 is now known to represent X pg of DNA, where X is the known DNA content value of the standard sample. Consequently, the quantity of DNA represented by peaks of previously unmeasured individuals or species along the x-axis can be calculated.

Choice of an appropriate DNA standard is crucial if errors are to be avoided in DNA content analysis and if confidence is to be placed in the results obtained (Johnston *et al.*, 1999). We considered *Hordeum vulgare* cv. sultan to be the most suitable standard for our studies. The mean nuclear DNA content of this species is 2C = 11.13pg (Johnston *et al.*, 1999) which lies close to, but does not overlap, the expected values of our study species, hence facilitating accurate determination of nuclear DNA content in our unknown samples.

Once the standard had been run on the cytometer and its' 2C peak adjusted to channel 200, the unknown sample was then run on its own. This allowed the 2C and 4C peaks

of the unknown samples to be identified in relation to those of the standard. The percentage CV for unknown samples' 2C peak was then checked and, assuming a ≤3.5% CV, a standard/unknown sample mix was run through the instrument a minimum of two times. The channel numbers of the 2C peaks for both the standard and unknown samples were recorded for all runs and subsequently tabulated for use in calculating the index of each run using the following equation:

Index = value for unknown sample / value for standard sample

The mean index value was then calculated and multiplied by the 4C DNA content value (in pg/nucleus) of the standard to give the 4C DNA content value of the unknown sample. Division of this figure by two provided us with the 2C DNA content of the previously unknown sample.

Statistical analysis: A minimum of two replicate measurements of DNA content were taken for each of the 180 individuals examined and the average 2C DNA content value was then calculated for each. Analysis of variance (ANOVA) was then performed on DNA content values among populations and associated standard error values were calculated.

A previous study by Huff & Palazzo, (Huff and Palazzo, 1998), used flow cytometry for the analysis of several fine fescue species, including *F. r. rubra* and *F. r. commutata*, in an attempt to clarify previous species and sub-species determinations based on chromosome numbers. Their study was based on 48 populations (accessions) taken from the 1989 National Turfgrass Evaluation Program at Rutgers University and the 1993 National Turfgrass Evaluation Program at the Pennsylvania

State University, both in the United States. Eight of these populations represented *F. r. rubra* and nine represented *F. r. commutata*. The mean and ranges in DNA contents for the two cytotypes reported by Huff & Palazzo in their study allow for comparison with our populations, once again via the performance of ANOVA.

***Appendix 6*: Nuclear DNA contents of *Festuca rubra* individuals from six sub-plots of the Park Grass Experiment, estimated via flow cytometric analysis.**

	Sub-plot					
Individual	**10b**	**16d**	**13a**	**13d**	**17a**	**17d**
1	13.34	13.38	13.00	14.06	13.79	13.20
2	12.75	13.5	13.08	13.32	13.33	13.66
3	12.97	13.34	13.71	13.70	13.31	13.85
4	12.87	13.59	12.52	13.86	13.26	14.33
5	13.33	13.82	13.16	13.59	12.85	14.81
6	13.03	13.39	12.45	13.76	13.87	12.78
7	13.72	13.61	11.82	14.11	13.57	13.48
8	14.52	13.82	13.31	13.70	13.76	14.02
9	15.30	14.07	13.31	14.22	13.14	14.39
10	15.68	14.35	12.06	14.07	13.37	15.16
11	13.08	13.1	13.15	14.07	12.79	12.73
12	13.18	13.47	13.58	13.76	13.31	12.99
13	13.35	13.7	13.74	13.85	13.25	13.62
14	13.70	13.99	13.36	14.03	12.76	14.71
15	14.07	14.28	12.71	13.98	13.37	15.63
16	13.54	13.15	12.57	13.87	14.10	12.86
17	13.54	13.54	13.00	13.58	12.89	13.3
18	13.54	14.08	12.99	13.72	13.02	13.75
19	13.54	14.64	13.79	14.22	13.23	13.97
20	13.54	15.05	13.54	13.86	13.13	14.78
21	14.24	14.09	13.82	13.54	13.19	14.08
22	13.09	14.37	13.84	13.96	13.01	14.25
23	13.92	13.99	13.34	14.4	13.37	13.30
24	13.56	14.1	13.72	13.14	13.43	13.92
25	13.83	13.57	12.31	14.17	13.2	14.2
26	12.49	13.87	13.98	13.7	12.85	14.09
27	13.23	14.52	13.68	13.53	13.15	14.05
28	12.87	13.75	11.90	13.6	12.82	13.63
29	12.61	14.15	11.93	14.2	13.32	12.82
30	12.61	13.15	13.51	14.11	13.25	13.84

Appendix 7: List of abbreviations.

PCR:	Polymerase chain reaction
ddH_20:	Double distilled water
MCD:	Mean character difference
TPD:	Total proportional dissimilarity
DCA:	Detrended correspondence analysis
PCA:	Principle component analysis
ITS:	Internal transcribed spacer
rDNA:	Ribosomal
DNA:	Deoxyribonucleic acid
ANOVA:	Analysis of variance
TEN:	Trout erythrocyte nuclei
PGE:	Park Grass Experiment
PHYLIP:	Phylogenetic inference package